CORRELATION OF COX-2 EXPRESSION IN COLORECTAL CARCINOMA WITH CLINICOPATHOLOGICAL FEATURES

DISSERTATION

SUBMITTED FOR

M.D IN PATHOLOGY

THE TAMILNADU DR.M.G.R MEDICALUNIVERSITY,

DEPARTMENT OF PATHOLOGY

PSG INSTITUTE OF MEDICAL SCIENCES & RESEARCH

PEELAMEDU, COIMBATORE – 641004

TAMILNADU, INDIA

TABLE OF CONTENTS

CERTIFICATE

This is to certify that the dissertation work entitled **"CORRELATION OF COX-2 EXPRESSION IN COLORECTAL CARCINOMA WITH CLINICOPATHOLOGICAL FEATURES"** submitted by **Dr. Manjula R.** is a work done by her during the period of study in the department from 31/05/2012 to 30/05/2015. This work was done under the guidance of **Dr. Sandhya V,** Professor, Department of Pathology, PSG IMS & R.

Dr. Prasanna N Kumar
Professor & HOD,
Pathology
PSGIMS & R
Coimbatore

Dr. S.Ramalingam
Principal
PSG IMS&R
Coimbatore

CERTIFICATE

This is to certify that the dissertation work entitled **"CORRELATION OF COX-2 EXPRESSION IN COLORECTAL CARCINOMA WITH CLINICOPATHOLOGICAL FEATURES"** submitted by **Dr. Manjula R** to The Tamilnadu Dr M.G.R Medical University, Chennai, for the award of the degree of Doctor of Medicine in Pathology, is a bonafide record of research work carried out by her under the supervision of **Dr.Sandhya V**, Professor of Pathology, PSG IMS&R. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Dr.Manjula R
Post Graduate,
Pathology
PSG IMS&R
Coimbatore

Dr.Prasanna N Kumar
Professor & H.O.D
Pathology
PSG IMS&R
Coimbatore

PSG Institute of Medical Sciences & Research

Institutional Human Ethics Committee

POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : psgethics2005@yahoo.co.in

November 06, 2012

To
Dr R Manjula
I year Post Graduate
Department of Pathology
PSG IMS & R
Coimbatore

Ref.: Your study entitled 'Correlation of COX 2 expression in colorectal carcinoma with clinicopathological features'

Ref.2: Our letter dated 21.09.2012
Documents submitted by you on 01.10.2012

Sub.: Ethics Committee Approval for the study

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on 21st September, 2012 in its expedited review meeting held at College Council Room, PSG IMS&R, between 9.00 am and 10.30 am, and discussed your application to conduct the study entitled:

"Correlation of COX 2 expression in colorectal carcinoma with clinicopathological features"

The following documents were received for review:

1. Duly filled application form
2. Confidentiality Statement
3. CV

After due consideration, the Committee has decided to approve the above study.

The members who attended the meeting held on 21.09.2012, at which your proposal was discussed, are listed below:

Name	Qualification	Responsibility in IHEC	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
Dr P Sathyan	DO, DNB	Clinician, Chairperson	Male	No	Yes
Dr S Bhuvaneshwari	M.D	Clinical Pharmacologist Member - Secretary	Female	Yes	Yes
Dr Sudha Ramalingam	M.D	Epidemiologist Alt. Member - Secretary	Female	Yes	Yes
Dr Y S Sivan	Ph D	Member - Social Scientist	Male	Yes	Yes

POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : psgethics2005@yahoo.co.in

Dr D Vijaya	Ph D	Member – Basic Scientist	Female	Yes	Yes

The approval is valid for one year.

We request you to intimate the date of initiation of the study to IHEC, PSG IMS&R.

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the full board review meeting scheduled on 30.11.2012.

Yours truly,

SECRETARY
PSG IMS & R
COIMBATORE-641 004
INSTITUTIONAL HUMAN ETHICS COMMITTEE

6.11.12

Dr S Bhuvaneshwari
Member - Secretary
Institutional Human Ethics Committee

CORRELATION OF COX-2 EXPRESSION IN COLORECTAL CARCINOMA WITH CLINICOPATHOLOGICAL FEATURES.

INTRODUCTION

Colorectal carcinoma is the fourth common cancer in the world and second most common cause of cancer related death[1]. Epidemiological studies have shown a lower incidence of colorectal adenomas and carcinomas in subjects who have taken NSAIDS (Non Steroidal Anti-Inflammatory Drugs) for a long time which suggest a pathogenic role for cyclooxygenase (COX -2) in colonic tumorigenesis. COX-2, an inducible isoform of cyclooxygenase is usually absent or present in low levels in normal colonic epithelium and is upregulated in colorectal carcinoma. Assessment of this molecular factor would therefore help in identifying the patients who are likely benefit from COX-2 inhibitor adjuvant therapy which attenuates the metastatic potential of colorectal carcinoma, thereby improving the prognosis. Literature search revealed no such study across the Indian population. Therefore the present study aims to evaluate COX-2 expression in colorectal carcinoma and to correlate it with clinicopathological features- age, sex, tumor location, size,

ACKNOWLEDGEMENT

It gives me an immense pleasure to express my sincere gratitude and heartfelt thanks to my guide **Dr. Sandhya V**, Professor of the Pathology, PSGIMS &R for her invaluable guidance, immense patience, timely advice and constant support.

I am thankful to our Head of The Department **Dr. Prasanna N Kumar**, Department of Pathology and I would like to extend my thanks to all faculty members and whom I have had the privilege to work with emeritus Professor **Dr. NirmalaV, Dr.Shanthakumari S** & **Dr. T M Subba Rao** and all assistant and associate professors for their support.

I am also thankful to my colleague and friends who have helped me to retrieve the tissue blocks & technical support

I am extremely grateful to my husband **G. Mohanraj** for his support & to my son Dakshin Pranesh Mohanraj for his understanding and to my parents and in laws for their prayers and blessings

I am thankful to our senior technician **Angeline Mary** and other staff members for their technical support

Last but not least, I want to thank all the patients whose data I used for my thesis.

CORRELATION OF COX- 2 EXPRESSION IN COLORECTAL CARCINOMA WITH CLINICOPATHOLOGICAL FEATURES.

ABSTRACT

Introduction : Colorectal carcinoma is the most common neoplasm of the gastrointestinal tract. COX-2 (cyclooxygenase-2) expression is upregulated in colorectal carcinoma. Therefore its assessment would identify patients amenable to adjuvant therapy. As no studies have been done across the Indian population as compared to western , the present study was done.

Aims and objectives: To study COX-2 expression in colorectal carcinoma and correlate it with clinicopathological features like age, sex, tumor location, size, degree of differentiation, depth of invasion, lymph node status and TNM stage.

Methods: 65 consecutive cases of colorectal adenocarcinoma between January 2009 to December 2013 were retrived from records of Pathology Department at PSG IMS&R. Immunohistochemical staining for COX-2 was done and correlated with age, sex, tumor location , size, degree of differentiation, depth of invasion, lymph node status and TNM stage.

Results : COX-2 was expressed in 86.2% of cases and negative in 13.8%. 90% of left side colonic carcinoma and 77.3% of right colonic carcinoma expressed COX-2. Among the lymph nodes with metastasis, 22.25% were COX-2 negative, 25% low positive and 47.7% were high positive . High positive

COX2 cases constituted 33.3% of stage I, 58.8% of stage II, 80% of stage III and 100% of stage IV tumors. About 56.6% of well differentiated, 66.6% of moderately differentiated and 100% of poorly differentiated carcinomas showed high COX-2 expression. The COX-2 overexpression was associated with advancing stage of tumor, more frequent lymph node and distant metastasis, decreasing degree of differentiation.

Conclusion : Determination of COX-2 expression in colorectal carcinoma gives prognostic information. COX-2 overexpression implicates advancing stage of disease. These patients can be treated with selective COX-2 inhibitors like Celecoxib, Rofecoxib, L-745,337 and SC 58125 as an adjuvant to chemo and radiotherapy.

Key Words: Colorectal carcinoma, COX2, COX2 inhibitor, Metastasis.

INTRODUCTION

Colorectal carcinoma is the fourth common cancer in the world and second most common cause of cancer related death[1]. Epidemiological studies have shown a lower incidence of colorectal adenomas and carcinomas in subjects who have taken NSAIDS (Non Steroidal Anti-Inflammatory Drugs) for a long time which suggest a pathogenic role for cyclooxygenase (COX -2) in colonic tumorigenesis. COX-2, an inducible isoform of cyclooxygenase is usually absent or present in low levels in normal colonic epithelium and is upregulated in colorectal carcinoma. Assessment of this molecular factor would therefore help in identifying the patients who are likely benefit from COX-2 inhibitor adjuvant therapy which attenuates the metastatic potential of colorectal carcinoma, thereby improving the prognosis. Literature search revealed no such study across the Indian population. Therefore the present study aims to evaluate COX-2 expression in colorectal carcinoma and to correlate it with clinicopathological features- age, sex, tumor location, size, depth of invasion, histological type, degree of differentiation, lymph node metastasis and stage of the tumor.

Aims and objectives

AIMS AND OBJECTIVES :

Primary Aim:

To study the COX-2 expression in colorectal carcinoma.

Secondary Aim :

To correlate the COX-2 expression with clinicopathological features – age, sex, tumor location, size, histological type, degree of differentiation, depth of invasion, lymph node metastasis and TNM (AJCC) stage.

Review of Literature

REVIEW OF LITERATURE

Incidence and disease burden

Colorectal carcinoma is the most common neoplasm of gastrointestinal tract. According to global cancer statistics 2002, Colorectal carcinoma is the fourth most common cancer worldwide and second most common cause of death[1] .They are most prevalent in North America, Europe, Australia and New Zealand compared to Asia and Africa[1]. In India, the incidence is 30- fold lower than the former group[1] . This geographical variation is due to different dietary, environmental factors. According to United States Surveillance Epidemiology & End Results (SEER) program, colorectal carcinoma accounts for 12% of all cancers with incidence rate of about 33.7 and 12.8 per one lakh for colon and rectal carcinoma respectively[2]. Mortality rate was about 15% according to American cancer society statistics(2000-2004)[2]. The incidence rate has been declining in the recent years considerably in North America due to modification of dietary habits and early screening[3]. Males are more frequently affected than females[1]. Incidence increases with age, most of the patients being above 40 years with a median age of 71 years according to SEER statistics. In patients younger than 50 years, family history is necessary in assessing the carcinoma risk, as 1% is associated with Familial Adenomatous Polyposis and 5% with Hereditary Non polyposis Colon Cancer (HNPCC) [1]. Location of colorectal

cancer in various sites of colon differs between male and female, and also reflect environmental, genetic factors and age of presentation[3] .

Location of tumor	Males	Females
Right colon	30%	40%
Left colon	30%	30%
Rectum	40%	30%

ETIOPATHOGENESIS :

The pathogenesis of colorectal cancer depends on environmental, diet and genetic factors, the polyposis syndromes being the most important ones.

Environmental factors :

Apart from diet, decreased physical activity, occupational exposure, alcohol, smoking, inflammatory bowel diseases are other factors having a role in the pathogenesis of colorectal carcinoma.

Dietary factors:

..... ***H***igh intake of fat and animal protein provides an excess source of calories resulting in weight gain. In addition, it also cause increase in the formation of hydroxyl radicals which in turn results in oxidative injury of

colonic epithelium predisposing to neoplastic progression [4,5]. It also increases bile acid and cholesterol synthesis, the toxic metabolites of which are carcinogenic [4].

Good intake of fruits, vegetables with high fibre content and micronutrients like carotenoids, flavinoids, ascorbate, isothiocyanate and phytic acid decrease the risk of developing colorectal cancer as these increase the defecation frequency and decrease mucosal contact time of potential carcinogens.

The consumption of vitamin A,C,D,E, calcium, selenium reduces the risk by their antioxidant action which prevent formation and neutralise the carcinogens [6,7]. In addition, calcium decreases the bile acids and fatty acids mediated injury of colonic epithelium by converting them into insoluble calcium soaps, thereby decreasing epithelial cell proliferation[7]. Selenium, a cofactor of glutathione peroxidase reduces oxidative damage of epithelium.

The low fibre diet decreases the stool bulk and alters the intestinal flora resulting in increased free radicals which remains in contact with colonic mucosa due to increased transit time, causing epithelial injury [4].

Alcohol consumption results in positive energy balance and abnormal DNA methylation[8]. Smoking, tobacco chewing, snuff and pipes on prolonged use is associated with microsatellite instability and act as a tumor initiator [9].

Obesity, sedentary occupation, excess energy intake, decreased physical activity in association with high fat intake is a risk factor. Increased physical activity cause stimulation of hormonal release, increase in peristalsis and neural reflex mechanism resulting in decreased mucosal exposure to carcinogens [10].

Individuals with prolonged exposure to metal dusts, plastics, organic solvents, fibreglass, fumes and asbestos are at increased risk for developing rectal cancer [1].

Intestinal factors :

Increased risk of cancer is seen in patients with diverticulosis, ulcerative colitis, Crohn's disease and adenomatous polyps [1]. Carcinomas occurring in association with Schistosoma Japonicum infestation are multicentric and present at an early age [1].

Hormonal factors :

Type 2 diabetes mellitus shows an increase in insulin resistance and glucose intolerance. Increased insulin levels in Type 2 DM promotes epithelial proliferation and inhibit apoptosis of colonic epithelial cells favouring tumorigenesis [11]. The increase in glucose level provides greater energy source for colonocytes.

Estrogen receptors are highly expressed in colonic tumors which implicate the protective role of estrogen.

Increased gastrin and growth hormone levels have been implicated in colorectal tumors. In colonic cancers, serum gastrin levels may be increased , probably due to autocrine secretion from tumor. The gastrin has growth promoting action. In persons with acromegaly, an increased growth hormone status resulted in increased cell proliferation and tumor formation.[1]

Others :

Radiation therapy for cancers of female genital tract and prostate increases the risk of colorectal carcinoma.

Surgical procedures like cholecystectomy, ileal conduits, gastric surgery for peptic ulcer may contribute to carcinogenesis. Epithelial proliferation occurs due to activation of fecal carcinogens by the diverted urine. Gastric surgery for peptic ulcer may alter the bile acid metabolism increasing unconjugated and secondary bile acids predisposing to carcinoma[1].

Predisposing host factors :

Among the endogenous factors, inflammatory bowel disease, genetic polyposis syndromes are important [2].

<u>Inflammatory bowel disease</u>

Inflammatory bowel disease is a long standing chronic inflammatory process which includes Ulcerative colitis and Crohn's disease. Numerous studies demonstrate an association between these diseases and colorectal carcinoma. The pathogenesis includes two hypothesis, one is a normal immune response to abnormal environmental stimuli while other is an abnormal immune response to normal stimuli [12].

IBD is more common around the third decade. In long standing IBD, with increasing duration of disease, the risk of developing precursor lesions like dysplasia or invasive carcinoma was higher [13]. Patients with extensive colon involvement upto or proximal to hepatic flexure are at greater risk for developing colorectal cancer [13]. The incidence begins to increase after 8-10 years of onset of the symptoms with an incidence rate of about 5-10% and 12-20% after about 20 and 30 years of disease respectively [14]. But when IBD is limited to left colon the risk of developing cancer increases after 15-20 years.

Patients with isolated ulcerative proctitis do not have greater risk for developing cancer , incontrast to patients with pancolitis [13]. Therefore routine

surveillance for dysplasia is recommended for patients with crohn's or ulcerative colitis. A routine colonoscopy every 1 or 2 years for lifetime is recommended [13].

Polyps :

Polyps of the colon are predominantly of epithelial origin, categorised as hyperplastic, adenomatous and inflammatory polyp.

Hyperplastic polyp:

About 25-30% of large intestinal polyps are hyperplastic in nature, grossly presenting as pale sessile nodule < 5mm in size with smooth glistening surface[1]. Three subtypes have been identified namely the Microvesicular hyperplastic polyp (MVHP), Mucin poor hyperplastic polyp (MPHP) and the Goblet cell hyperplastic polyp (GCHP).

Hyperplastic polyps in general has low malignant potential. However mutations have been identified in MVHP and GCHP. The microvesicular hyperplastic polyp is associated with V600E BRAF mutation and activation of MAPK (MAP Kinase) pathway[15]. The surface colonocytes, in the absence of apoptosis persist, resulting in serrated morphology. The goblet cell type hyperplastic polyps are associated with KRAS mutation [15]. Characteristic microscopic features include marked luminal serration, mitosis and mild

nuclear atypia at the crypt base, surface maturation and thicker collagen table [2].

Adenomatous polyps:

Adenomas are common premalignant lesions of colon. They are classified as conventional, serrated or flat types. Conventional adenomas are subtyped as tubular, villous or tubulovillous adenomas [2]. Polyps occuring in the context of hereditary colon cancer syndromes was usually multiple and present at a younger age group [1]. Among various colonic polyposis syndromes, Familial adenomatous polyposis with its attenuated form, Gardner's syndrome, Turcot's syndrome, MYH associated polyposis and Lynch syndrome are most common [2].

Familial adenomatous polyposis:

Familial adenomatous polyposis has an autosomal dominant inheritance with APC (Adenomatous Polyposis Coli) gene mutation (a tumor suppressor gene). In addition KRAS and p53 mutations are also encountered. It is characterised by presence of more than 100 tubular adenomatous polyps involving the entire colon which by definition harbours low grade dysplasia [2]. Cyclooxygenase 1 expression has been seen in stromal cells in FAP at an early stage followed by COX-2 expression resulting in PGE2 mediated growth of the

polyp [16]. Thus in addition to COX-2, COX-1 also plays a role in the initial stages of development of polyp [16].

Gardner's syndrome, an autosomal dominant condition with APC gene mutation, develop in addition to adenomatous polyps, desmoid tumor and osteomas.

Turcot's syndrome shows coexistence of FAP or Hereditary nonpolyposis colon cancer with central nervous system tumors. APC gene mutation or germline defect in DNA mismatch repair genes have been reported [2].

Attenuated FAP also known as hereditary colon cancer syndrome with mutation similar to FAP. Morphologically the condition differs from conventional FAP, by the presence of less than 100 polyps and the risk of developing colorectal carcinoma at an older age group [2].

MYH associated polyposis is an autosomal recessive syndrome with mutation in Mut Y homologue (MYH) gene which encodes an enzyme responsible for preventing mutation after DNA damage due to oxidation. Morphologically more than 30 adenomatous polyps are seen with a high risk for colorectal carcinoma.

Mutation of DNA mismatch repair genes (hMLH2, hMSH6, hMSH2, hPMS2) resulting in microsatellite instability is seen in Hereditary Non

Polyposis Colorectal Cancer Syndrome (HNPCC), also known as Lynch syndrome . The polyps are of adenomatous type, usually less than 10 in number with high grade dysplasia and increased risk for malignant transformation.

Morphology of adenomas :

Grossly, the adenomas may be pedunculated, flat or sessile. Tubular adenomas are pedunculated, small with surface lobulation while villous adenomas lack a stalk, present as flat or lobulated, sessile, large shaggy mass and have a broad base. Tubulovillous adenomas show overlapping features of tubular and villous architecture ranging between 20-79% [1].

Microscopically ***tubular adenomas*** show increase number of crypts with dysplasia of surface epithelium, focal tubular dilation and mixed inflammatory cells in the lamina propria. The villous adenoma consist of more than 80% finger like fronds with a core formed by lamina propria, lined by dysplastic epithelium. All type of adenomas are composed of mixture of absorptive cells, intermediate cells, goblet cells, paneth cells and endocrine cells. Flat adenoma and villous adnenomas are most commonly associated with high grade dysplasia. Hypersecretory and clear cells are other rare types of adenomas [1].

Serrated adenomas are less frequently reported type of polyps. They may be single or multiple. Histologically serrated glands are lined by less mature

stratified dysplastic cells with surface mitosis, papillary tufting and eosinophilic cytoplasm with increased nucleo-cytoplasmic ratio and prominent nucleoli.

Hyperplastic and serrated polyps constitute 7% of premalignant lesions in IBD [13].

Dysplasia associated lesions :

Dysplasia can be associated with both inflammatory bowel disease and polyps. In 1981, Blackstone et al, described the term DALM or Dysplasia Associated Lesion or Mass in ulcerative colitis. The DALMs can morphologically resembles sporadic adenoma which was termed as adenoma like dysplasia associated lesions or masses.

The differentiating features between DALMs and sporadic adenoma is that the former shows bottom up growth pattern with dysplastic cells at the bottom of the crypts in the background of chronic active inflammation, while the latter shows top-down growth pattern with luminal surface of crypts lined by dysplastic cells [13].

PATHWAYS OF COLORECTAL CARCINOGENESIS :

The molecular changes in colorectal adenocarcinoma are heterogenous including genetic alteration and epigenetic abnormalities [4]. The two distinct forms of genomic instabilities are chromosomal instability and microsatellite instability. Till date at least three distinct pathways of colorectal carcinogenesis have been identified [2].

1.APC / β catenin pathway

2. Microsatellite Instability Pathway

3.Serrated neoplasia pathway OR CpG Methylation pathway

1. APC / β catenin pathway :

APC gene mutation was identified commonly in individuals with familial adenomatous polyposis [(17,18)]. Vogelstein et al described it as a type of chromosomal instability with multistep progression from hyperproliferative epithelium to adenoma of increasing size and dysplasia, finally resulting in invasive carcinoma [19]. The members of this pathway are APC, KRAS, p53, β-catenin, DCC and DPC4 genes [4].

APC gene is located in chromosome 5 , undergoes germline mutation in FAP patients and somatic mutation in sporadic adenocarcinomas [20]. The APC

gene is a most important component of Wingless (WnT) signalling pathway. It act as " Gate keeper gene" with negative regulation of colonic epithelial proliferation in colorectal neoplasia [2]. Most colorectal cancer and adenoma patients have at least one allele mutation according to knudson hypothesis [4]. In tumorigenesis, the second allele also mutated results in loss of tumor suppressor effects [20].

β−Catenin can be dysregulated by APC gene mutation or CTNNB1 mutation (β catenin gene) [17]. Normally APC gene causes ubiquitin mediated degradation of β catenin in cytoplasm, thereby preventing it from entering the nucleus where it induces cell proliferation by interacting with transcription of Myc and cyclin D [21].

KRAS are protooncogenes having role in signal transduction from growth factor receptors. Mutation cause autonomous cell proliferation. KRAS mutations follow loss of APC in the adenoma-carcinoma pathway [19].

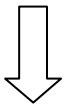
p53 gene, located on chromosome 17 is a tumor suppressor gene. The most common functions are control of cell proliferation, DNA repair and programmed cell death. Arrest of cell cycle at G1 phase, inhibition of cyclin CDC2 or cyclin CDK complexes are also the functions of p53. Loss of wild type p53 due to mutation or deletion therefore is a major step in carcinogenesis because of loss of check point in cell cycle and absence of DNA repair. This

results in amplification of unrepaired DNA and upregulated cell growth. p53 overexpression is an important predictor of survival status in patients with lymph node positive colorectal carcinoma [1].

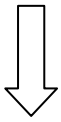
Loss of heterogenesity of DCC (Deleted in Colon Cancer) and SMAD4 or DPC4 (Deleted in Pancreatic Cancer, locus 4) on chromosome 18 is commonly seen in colorectal carcinoma. These genes encode TGF-β signalling proteins which normally inhibits cell cycle [4] and the mutation of TGF-β causes increased cell proliferation. Loss of SMAD4 gene is seen in late stages, suggesting it as a late event in carcinogenesis [4].

Sequence of events of APC /β catenin pathway [4]

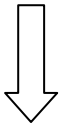
Normal colon with germline or somatic mutation of single allele of APC at chromosome 5.



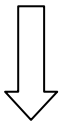
Hypermethylation and inactivation of second allele of the APC gene results in increased β-catenin levels. The mucosa is put to increased risk for tumor formation.



Subsequent KRAS and p53 mutations results in increased cell proliferation and inhibition of apoptosis



ADENOMAS develop. COX-2 overexpression occurs (discussed in detail later in the review of literature)



Together with loss of heterogenesity of SMAD4 &2 and increased telomerase activity, progression from adenoma to carcinoma occurs.

2. Microsatellite instability pathway:

Mismatch repair genes correct the genetic alteration occurring during DNA synthesis. In conditions with DNA mismatch repair deficiency, mutation accumulate in microsatellite repeats which is referred to as MICROSATELLITE INSTABILITY (MSI). Many of the microsatellites are located in noncoding regions.

When the microsatellites located in promoter or coding region of the genes like TGF-β type II receptors, BAX proteins become unstable, uncontrolled cell growth and proliferation occur [4]. These promoter regions are responsible for regulation of cell growth by encoding the TGF-β type II receptor and BAX proapoptotic protein. Normally TGF-β inhibit proliferation, so in the presence of TGF-β mutation, uncontrolled cell growth occurs with increased survival due to BAX loss. Other mutations in BRAF and CpG hypermethylation also develop in this defect [4].

MSI was initially discovered in HNPCC, most common cause of right sided colonic tumor with a higher tendency to be of mucinous, poorly differentiated type. There are three categories of microsatellite alteration

(a) MSS(Micro Satellite Stable),

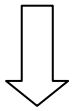
(b) low level MSI

(c) high level MSI.

MSI-low level occurs with <30% unstable microsatellite loci and MSI-high level show >30% microsatellite instability loci [22].

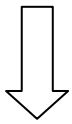
Sequence of the pathway [22]

In normal colon with germline or somatic mutations of mismatch repair genes

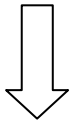


Alteration in second allele by Loss of Heterogenesity by methylation or

mutation of MLH_1, MSH_2,MSH_6, PMS_1 and PMS_2.

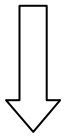


Microsatellite instability is initiated



Accumulation of gene mutations results in uncontrolled cell proliferation and

inhibition of apoptosis by alteration in TGF-β RII, BAX, BRAF and others.



COLORECTAL CARCINOMA

3. SERRATED NEOPLASIA PATHWAY OR THE CPG METHYLATION PATHWAY

Jass et al, proposed that carcinomas arising by the serrated pathway are heterogenous groups, the molecular profiles of which have been subtyped as follows [15,23].

1.BRAF mutant, CIMP-H with MSI-high level.

2.BRAF mutant, CIMP-H with MSS.

3.Kras mutant, CIMP-L, MSS.

[CIMP-H or L : CpG Island Methylation Phenotype-High level or Low level, MSI: MicroSatellite Instability, MSS: MicroSatellite Stable].

1.BRAF mutant / CIMP-H / MSI-H :

The colorectal carcinomas with this genetic profile are more common (9-12%) in elderly women, situated in right colon, and are at high tumor stage without lymphnode or distant metastasis at presentation. The sessile serrated adenomas, a precursor lesion undergoes progressive methylation and silencing of MLH_1 (key promoter region) resulting in high grade dysplasia and microsatellite instability.

Prognosis is favourable. However these tumors are resisitant to most non-surgical treatments like chemotherapy including 5-flurouracil, monoclonal EFGR inhibitors (cetuximab & panitumumab) [15].

2.<u>BRAF mutant / CIMP-H / MSS:</u>

Sessile serrated adenomas with dysplasia in the absence of loss of MLH_1 gene are proposed precursor lesion of this subgroup of mutation (5-10%). Compared to conventional colorectal carcinomas, these tumours are often poorly differentiated and have higher incidences of tumor budding, lymphovascular and perineural invasion with lymphnode metastasis.

The genetic abnormalities includes methylation of different promoter region and silencing of p16, Tp53 and WnT pathway genes. These tumors have a poor prognosis.

3. <u>KRAS mutant / CIMP-L / MSS</u> :

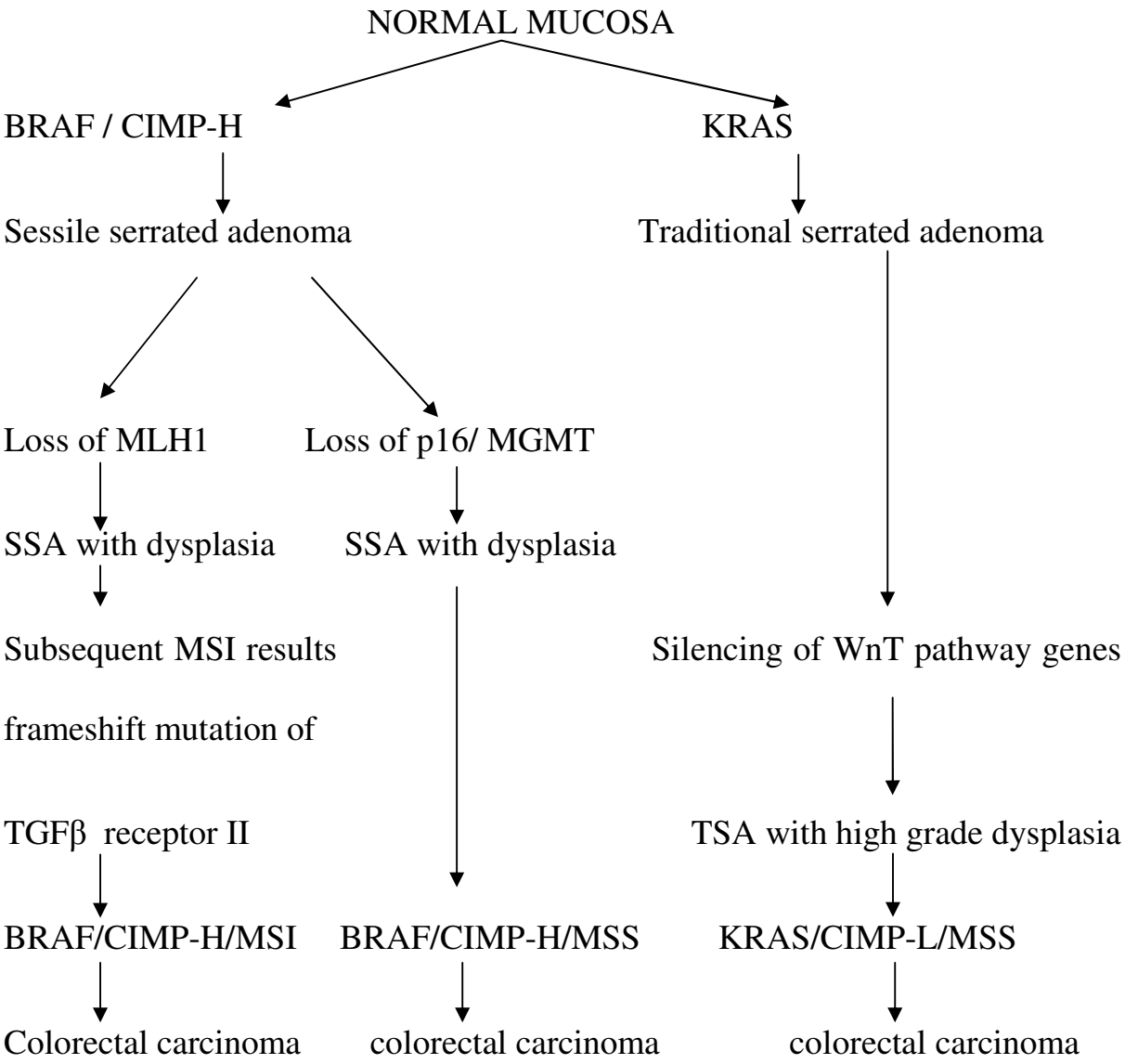
KRAS mutation with microsatellite stable subgroup is the largest group (15-20%) among other mutants. Serrated tubulovillous adenoma are the precursor lesions progressing via this pathway. Low level CIMP segregate these tumors as a subtype with poor prognosis [15].

Serrated morphology carcinoma :

Serrated carcinoma refers to the tumors with distinctive serrated morphology and derived via serrated neoplastic pathway. These carcinomas initially described by Jass and Makinon et al have defined the histological criteria. The World Health Organisation has recognised serrated carcinomas as a distinct subtype of colorectal carcinoma [15].

These serrated carcinomas are frequently seen in women and the common sites are caecum, ascending colon followed by rectum [24].

SEQUENCE OF THE SERRATED PATHWAY [15]



Comparing these three molecular profiles, the BRAF/ CIMP-H/ MSI has good prognosis but resistance to chemotherapy, while the remaining two mutations has poor prognosis but are sensitive to chemotherapy [15].

INFLAMMATORY BOWEL DISEASE ASSOCIATED CARCINOGENESIS :

Inflammatory Bowel Disease (IBD) associated carcinogenesis is a multistep process, starting from inflamed regenerative epithelium to hyperplasia to flat dysplasia and ends with invasive adenocarcinoma. The interaction of inflammatory cells like neutrophils and macrophages with colonic epithelium plays a key role in IBD-induced carcinogenesis. Various factors like reactive oxygen/nitrogen overproduction, cytokines, growth factors and arachidonic acid metabolites, activated inflammation associated signal pathways, together with immune dysfunction contributes to carcinogenesis in IBD.

Similar to sporadic colorectal cancer the characteristic molecular abnormalities are mutation in oncogenes, tumor suppressor genes and DNA repair genes with genomic instability[13]. Chronic inflammation results in oxidative stress which plays a characteristic important role in this carcinogenesis. The oxygen and nitrogen free radicals bind to target protein, DNA, RNA and result in genetic alterations, aberrant methylation and genomic insatability.

Free radicals cause chromosomal instability secondary to DNA translocation/ amplification/ deletion/ breakage/ telomere damage and p53 mutations [25]. In ulcerative colitis, telomere shortening has been linked with

dysplasia[25]. Interaction of free radicals with the cell membranes leads to lipid peroxidation results in mutation of p53 tumor suppressor gene. Aberrant activation of arachidonic acid pathways and COX-2 overexpression are important in IBD associated carcinogenesis [26]. In ulcerative colitis, COX-2 expression elevated along with mRNA expression in inflamed mucosa, dysplasia and in carcinomas. COX-2 activates procarcinogens , increases free radical production and angiogenesis [26].

IBD associated carcinogenesis and sporadic carcinogenesis shows similar molecular changes. The difference in the timing and frequency of the alterations distinguish both types. p53 is a key factor in IBD associated carcinogenesis and KRAS mutation, loss of APC genes are less common and occur late in carcinogenesis. Genes with methylation of CpG islands result in dysplasia throughout the mucosa with ulcerative colitis.

Aneuploidy is usually seen in patients with more than 10 years disease duration and is associated with dysplasia [15]. Fluroscent In-Situ Hybridisation (FISH) analysis shows ulcerative colitis associated dysplasia or carcinoma exhibiting monosomies and polysomies.

Microsatellite instability was frequently associated with ulcerative colitis patient with dysplasia and carcinoma. p53 mutation in adjacent mucosa, APC and KRAS mutation are less frequently associated with carcinogenesis in the

later stages of ulcerative colitis indicating that KRAS play a role at the later stages of carcinogenesis.

CpG island methylation with hypermethylation of cyclin dependent kinase inhibitors 2A (CDKN2A) or p16 and p14 are common in ulcerative colitis associated adenocarcinoma. The p14 hypermethylation was detected in 50% cases .

In sporadic cancers the precursors are tubular adenoma. In contrast, the dysplasia in IBD are polypoid or flat and localised or diffuse. The concept of "Field Cancerisation" describes that the entire epithelium of upper aerodigestive tract has increased risk for premalignant lesions in IBD due to multiple genetic abnormalities [27]. This concept was widely accepted for other organs including colon, importantly in IBD associated dysplasia. This clonally derived mutant cells with indistinguishable histological features are seen in inflamed segment of IBD. For example the mutant TP53 or KRAS is detected across nondysplastic crypts as well as entire neoplasm [27].

Low grade dysplasia is associated with very short telomerases, high level of senescences together with DEC1(Deleted in Esophageal Cancer 1) overexpression. Above pattern is reversed in high grade dysplasia, an important features in IBD associated carcinogenesis [13].

The level of expression of an enzyme named α-methylacyl coenzyme A racemase (AMACR) ranging from negative in normal epithelium to high in invasive carcinoma. Hence p53 and AMACR coexpression are demonstrated in IBD associated carcinogenesis [15].

CLINICAL FEATURES :

Colorectal carcinomas shows wide range of clinical features from altered bowel habits, pain and tenesmus to surgical emergency like intestinal obstruction [17,18]. The clinical symptoms differ depending on the location and stage of the tumor [1]. Generally all patients have loss of weight, loss of appetite malaise, weakness and fatigue.

Right side colon cancers are often polypoidal and patients remain silent because of the soft stool consistency in this region or have features secondary to iron deficiency anemia, cardiac failure or angina pectoris. When the tumor obstructs the lumen of appendix, patients may manifest with symptoms similar to appendicitis. Most of the caecal tumours are however silent, present as an abdominal mass with metastasis [1].

Left sided colon cancers frequently presents with altered bowel habits like diarrhoea, alternating with constipation and incomplete rectal emptying sensation. In advanced stage, napkin ring like constriction can cause,

constipation and symptoms of obstruction . In case of obstruction, the proximal colon undergoes ischemic change and rupture [1].

Bleeding is a frequent finding in rectal carcinoma [1]. In advanced stages irrespective of the location , the tumours present with significant abdominal pain, perforative peritonitis and colo-colic fistula [1].

MORPHOLOGY:

Gross appearance of colorectal carcinoma depends on the stage of disease [2].

Small carcinomas present as red, granular, elevated circumscribed lesions resembling adenoma [1]. Aberrant crypt foci (ACF) is the earliest morphological change in the epithelial neoplasms of the colon [1].

Large carcinomas are categorised into four types:

(a) ***Polypoidal type growth pattern***:

It is most commonly seen in caecum and ascending colon presenting as a bulky mass [2]. Also referred to as fungating or exophytic pattern they are characterised by intraluminal growth with papillary surface and areas of ulceration. When seen in the caecum, they are

usually asymptomatic until the symptoms of anemia occurs due to chronic blood loss [1].

(b) ***Ulcerated or excavated type***:

It is an infiltrating type with elevated edges and intramural growth[18].

(c) *Annular or constricting type*:

It is the circumferential involvement of colon resulting in napkin ring constriction as seen by double contrast study [2]. The colon proximal to the constriction shows attenuation of mucosal folds and dilatation [2]. Both the ulcerated and annular types are commonly seen in descending and transverse colon [18].

(d) *Diffuse infiltrative or linitis plastica*:

There is diffuse flattening and thickening of mucosa with more of intramural spread in this growth pattern[2]. All types are relatively homogenous with areas of necrosis and infiltration upto the serosa resulting in retraction of serosal surface [2]. Tumors with high mucin contents appear gelatinous and glistening with separation of bowel layers [17].

MICROSCOPIC APPEARANCE:

Adenocarcinoma is the most common type of colorectal cancer. The tumor cells in colorectal carcinomas are arranged in glandular pattern and less commonly as single infiltrating cells in the poorly differentiated tumors. The surface of the tumors show papillary or villous pattern of arrangement [17].

The tumor cells are pleomorphic, columnar cells with increased nucleo cytoplasmic ratio, eosinophilic cytoplasm and conspicuous nucleoli. Admixture of goblet cells, neuroendocrine cells and paneth cells are also encountered. The tumor confined to mucosa is grouped as intramucosal and further invasion towards the serosa increased the tumor staging.

Inflammation and desmoplastic reaction are often seen in the surrounding stroma. Hyperplastic changes like taller and tortuous glands are most commonly seen at the edges of the tumor. Lymphovascular and perineural invasion has prognostic significance [17].

Adenocarcinomas are graded on the basis of extent of glandular differentiation into (1) Well, Moderately and Poorly differentiated or (2) Low grade [well and moderately differentiated] and High grade [poorly and undifferentiated]. More than 95%, 50-95% and 5-50% of glandular differentiation are seen in well, moderately and poorly differentiated carcinomas respectively[18]. Tumors with less than 5% glandular differentiation are

categorised as undifferentiated type [18]. These tumors have DNA mismatch repair deficiency and poor prognosis [2].

***Other types of carcinomas*:**

Mucinous carcinoma:

Mucinous carcinoma are most frequently seen in young patients with Hereditary Non Polyposis Colon Cancer syndrome[2]. The Conventional adenocarcinomas can have significant mucinous component. WHO recommends the term mucinous carcinoma when > 50% of tumor cells produce mucin [18].

The tumor epithelium are in acinar, clumps or single cell arrangement and these cells are freely floating in pools of mucin [18]. Microsatellite instability and previous villous adenomas are the most common associated features [1]. Mucinous carcinomas have very poor prognosis due to advanced stage at presentation, extensive lymphnode and pericolonic involvement [17].

There are two subtypes, colloid and signet ring cell carcinoma.

In colloid carcinoma, malignant disrupted glands are seen floating in pools of mucin. Association with HNPCC is frequently seen [1].

Signet ring cell carcinoma is a rare tumor seen in young patients with high microsatellite instability [17]. Microscopically, the criteria for diagnosis is

the presence of >50% of cells with prominent intracytoplasmic mucin [18]. These cells can be distributed in mucin pools or diffusely infiltrating the bowel wall [18]. The prognosis is poor due to advanced stage and frequent metastasis [17].

***Linitis plastica or Diffuse carcinoma*:**

Non signet ring cell carcinomas may present with diffuse growth pattern of neoplastic cells [2]. Marked desmoplasia results in thick rigid colon grossly [1]. Left colon is the most common site for this subtype with poor prognosis [1].

Undifferentiated carcinoma:

The rare undifferentiated type are grossly seen as bulky soft mass with extensive necrosis[2]. According to WHO, the carcinomas with no gland formation or less than 5% gland formation are described as undifferentiated carcinoma [18].

Histologically, the neoplastic cells are seen in sheets, cords and trabecular pattern with extensive necrosis and absence of desmoplasia . These tumors have DNA mismatch repair deficiency and poor prognosis [2].

Medullary carcinoma :

Medullary variant is more frequently seen in the right colon with associated Microsatellite Instability[17]. Microscopically, the malignant cells are arranged in sheets with abundant eosinophilic cytoplasm, vesicular nuclei, prominent nucleoli and characteristic lymphocytic infilteration [18]. Prognosis is favourable compared to poorly or undifferentiated carcinoma [18].

Small cell carcinoma :

Adenocarcinomas can exhibit neuroendocrine differentiation ranging from scattered endocrine cells, mixed composition to predominantly small cell (neuroendocrine) carcinomatous pattern. Microscopically it is characterised by presence of sheets of small cells with hyperchromatic nuclei. Foci of glandular differentiation can be present. Prognosis is poor with early lymphnode and distant metastasis [17].

***Adenosquamous carcinoma* :**

This is an unusual type of carcinoma, presents in young patients in advanced stage [1]. These tumors are usually associated with paraneoplastic secretion of parathyroid hormone and hypercalcemia [2]. Microscopically, both

squamous carcinomatous and adenocarcinoma components are present in separate areas or admixed [18]. Prognosis is poor compared to conventional adenocarcinoma [1].

***Squamous cell carcinoma* :**

Pure SCC is very rare in large bowel, caecum being a common location [18]. Criteria to diagnose primary squamous cell carcinoma of large bowel are:

(a) No evidence of squamous cell carcinoma in any other primary sites which could provide a metastatic source or direct extension.

(b) No continuity with the anal squamous epithelium.

(c) No evidence of associated squamous lined fistula in the affected bowel segment

(d) No glandular differentiation.

The prognosis is worse than adenocarcinoma [2].

Spindle cell or Sarcomatoid carcinoma:

Extremely rare tumor of elderly persons. Grossly present as bulky tumor with abundant hemorrhage. Microscopically, biphasic growth pattern is seen composed of epithelial and mesenchymal components. The mesenchymal component may be smooth muscle, osseous or cartilaginous type. The tumor has TP53 mutation in both epithelial and mesenchymal component. Highly aggressive tumor with poor prognosis [2].

***Serrated adenocarcinoma* :**

Serrated adenocarcinoma, a recently recognised entity arises from serrated or hyperplastic polyps. Pathogenesis reveals high degree of methylation and low or high level microsatellite instability [28].

Microscopically, tumor cells are arranged in serrated, trabecular or mucinous growth pattern with abundant eosinophilic cytoplasm, preserved nuclear polarity, condensed chromatin without any necrosis. The characteristic microscopic appearance has been described earlier.

Rare variants:

Apart from above mentioned types, giant or pleomorphic cell, clear cell, basaloid, hepatoid, paneth cell rich (crypt cell carcinoma), oncocytic and carcinosarcoma are also reported [18.]

STAGING OF COLORECTAL CARCINOMA [17]

The different staging systems for colorectal carcinoma include Duke's, Astler and Coller and American Joint Cancer Committee (TNM) staging. The aim of all staging systems is to predict the prognosis and to guide treatment.

DUKE'S STAGING :

In 1932, Duke established a staging system

A – Tumor confined to mucosa and submucosa

B - Tumor extend into bowel wall

C - Tumor extend beyond bowel wall with lymph node metastasis

ASTLER COLLER STAGING :

It is a modification of Duke's staging with the addition of stage D to include tumors with distant metastasis :

A-Tumor confined to mucosa and submucosa

B-Tumor invades into muscularis propria with no lymph nodes

B1-Tumor invade into muscularis propria

B2-Tumor invade through muscularis propria into serosa

C-Tumor invades into muscularis propria with lymph node deposits

C1- Tumor invade into muscularis propria with lymph node metastasis

C2-Tumor invade through muscularis propria into serosa with lymph node metastasis

D-Tumor with distant metastasis

AMERICAN JOINT CANCER COMMITTEE (TNM) Staging:

Primary tumor (T)

Tx – Primary tumor cannot be assessed

T0 – No evidence of primary tumor

Tis – Carcinoma in situ (Intraepithelial or invasion of lamina propria)

T1- Tumor invades submucosa

T2 – Tumor invades into muscularis propria

T3 – Tumor invades through the muscularis propria into subserosa or into nonperitonealised perirectal or pericolic tissues

T4 – Tumor directly invades other organs or structures or perforates the visceral peritoneum or tumor adherent to other organs.

N :Regional lymph nodes:

Nx – Regional lymph nodes cannot be assessed

N0 – No regional lymph node metastasis

N1 – Metastasis in 1 to 3 regional lymph nodes

N2 – Metastasis in 4 or more regional lymph nodes.

Distant metastasis:

Mx – Presence of metastasis cannot be assessed

M0 – No distant metastasis

M1 – Distant metastasis

STAGE GROUPING

STAGE	T	N	M
STAGE 0	Tis	N0	M0
STAGE I	T1 T2	N0 N0	M0 M0
STAGE II A	T3	N0	M0
STAGE IIB	T4	N0	M0
STAGE III A	T1-T2	N1	M0
STAGE III B	T3-T4	N1	M0
STAGE III C	Any T	N2	M0
STAGE IV	Any T	Any N	M1

The TNM staging recommended by AJCC (American Joint Committee) has replaced the Dukes and Astlers Collers staging system. The limitations of different staging systems compared to the AJCC (TNM) staging can be assessed from the comparison table below:

STAGING	T	N	M	Duke's staging	Astler coller's Staging
0	Tis	N0	M0	-	-
I	T1 T2	N0 N0	M0 M0	A A	A B1
IIA	T3	N0	M0	B	B2
IIB	T4	N0	M0	B	B3
III A	T1-T2	N1	M0	C	C1
III B	T3-T4	N1	M0	C	C2/C3
III C	Any T	N2	M0	C	C1/C2/C3
IV	Any T	Any N	M1	-	D

In Astler collar, tumor involving mucosa alone is graded as A, but in Duke's staging system , stage A are tumors involving mucosa and submucosa and in TNM, stage I are the tumors with mucosa, submucosa and / or muscularis propria involvement. Other stages are more or less equally categorised.

In TNM staging, the nodal involvement is subgrouped according to the number of lymph nodes with metastasis, while in the other staging systems only the presence or absence of lymphnode involvement is taken into consideration.

PROGNOSTIC FACTORS IN COLORECTAL CARCINOMA: [17]

1.***Age and sex***:

Tumors seen in very young and very old patients have poor prognosis. In very young patients, delay in diagnosis results in advanced stages. Association with ulcerative colitis is often present and are histologically of signet ring and mucinous carcinoma type. Females have good prognosis than males.

2. ***Tumor location:***

Studies reveals that lesion in left colon have better prognosis than right colon and rectum while the contrary has been observed in other studies.

3. ***Tumor multiplicity***:

Both synchronous and metachronous malignancies have similar survival rate like solitary adenocarcinoma.

4. ***Local extent of tumor:***

Polyp with focal microscopic carcinoma confined to mucosa and submucosa has excellent prognosis. Tumors with extension beyond the wall with regional lymph node metastasis have poor prognosis.

5.***Tumor size:***

A minor relationship was noted between tumor size and lymph node metastasis, prognosis.

6. ***Tumor edge:***

Adenocarcinoma with nonpolypoidal edge have worse prognosis than polypoidal carcinoma.

7. ***Obstruction and perforation:***

Both occur when there is extensive infiltration of bowel wall and it indicates worse prognosis.

8. ***Tumor budding:***

Tumor cells singly or >5% cells clusters at the invasive tumor front is a strong prognostic indicator for poor outcome.

9. ***Microscopic type:***

Among the various types of adenocarcinoma, mucinous, signet ring and anaplastic carcinoma have worse prognosis while medullary carcinoma has better prognosis.

10. ***Tumor margin and inflammatory reaction:***

Tumor with pushing type margins and marked lymphoplasmacytic infilterate at the interphase between tumor and adjacent bowel tissue have a better prognosis. Presence of eosinophils and dendritic cells in the stroma also

associated with good prognosis. But the presence of mast cells indicate a comparatively poor prognosis.

11. ***Lymph node involvement*:**

The extent of lymphnode metastasis determines the survival rate of the patients. Greater, the number of lymph nodes involved, the worser is the prognosis. Micrometastasis of lymph node detected by IHC(CK 20) or PCR (for CEA) have prognostic significance.

12. ***Tumor angiogenesis :***

Tumors with significant angiogenesis have high chance of recurrence, hence decreased survival rate.

13. ***Tumor thickness:***

Increased thickness of tumor in central depressed region correlates with lymphnode and distant metastasis and an unfavourable prognosis.

14. ***Angiolymphatic invasion:***

The tumor invading veins particularly extramural vessels, is associated with more frequent distant metastasis and decreased survival rate. Lymphatic vessel invasion has adverse prognosis if patients have stage III disease.

15. ***Perineural invasion:*** Perineural invasion has unfavourable prognosis.

16. ***Pericolonic tumor deposits:*** They are associated with poor prognosis.

17. ***Surgical margin:***

Presence of tumor < 2mm from the radial margin is a bad prognostic indicator with increased local recurrence.

18. ***Microscopic grading:***

The less differentiated tumors have poor prognosis. The grading is determined by worst pattern rather than the predominant one.

19. ***Pattern of lymphnode reaction:***

The regional lymphnodes may show evidence of cell mediated immune response characterised by paracortical expansion with immunoblasts and sinus histiocytosis. These patients has better survival rate than others without any change.

20. ***Staging of tumor:***

The patients in advanced stage of the tumor have poor prognosis.

21. ***Serum CEA levels***:

Increased serum carcinoembryonic antigen about more than 5 ng/ml have an adverse prognosis.

22. ***Mucin related antigens:***

The colorectal carcinomas with mucin associated antigens like sialyl-Tn and sialyl-lewis expression have aggressive outcome. MUC1, is an independent prognostic factor associated with high chance of progression of the tumor.

23. ***HLA-DR and BCl-2 expression***:

Both HLA-DR and BCl-2 expression are associated with good prognosis.

24. ***TGF-β Mutation***:

TGF-β type II receptor mutation with high levels of Microsatellite Instability have favourable prognosis.

25.***Oncogenes and tumor suppressor gene expression:***

Presence of MSI is associated with better patient survival. On the other hand, KRAS mutation, overexpression p53 and lack of p27 expression are associated with poor prognosis.

26. ***Allele loss of chromosome 18q:***

Loss of chr18q allele results in unfavourable prognosis. But retention of the alleles in microsatellite stable tumors indicate favourable outcome with good response to adjuvant chemotherapy in stage III carcinoma.

27. ***DNA ploidy:***

In rectal carcinoma, tumors with aneuploidy have increased risk of recurrence and unfavourable prognosis.

28. ***pRb and p16***:

Poorer outcome was seen in patients with aberrant expression of pRb and p16.

29***. Claudin 1:***

Claudin is a tight junction associated protein. Loss of claudin results in poor prognosis with high chance of recurrence.

30. ***Fascin:***

Immunohistochemical detection of increased fascin expression indicates decreased survival rate.

WHAT IS CYCLOOXYGENASE ?

Cyclooxygenase (COX) or prostaglandin H_2 synthase (PGHS) is the enzyme that catalyses the first two steps in the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid (AA). These include the oxidation of AA to PGG_2 (hydroperoxy endoperoxide) and reduction to PGH_2 (hydroxy endoperoxide) .

Two isoforms of this enzyme exist : COX-1 and COX-2 [29]. The PGH2 is then converted to PGE2, PGF2 , prostacyclin, PGD2 and thromboxane by specific enzymes as shown in the flow chart below (Fig 1) [30] .

Cyclooxygenase-1 (COX-1) was first obtained from bovine vesicular glands in 1976 [31] and COX-2 was discovered by Daniel Simmons laboratory at Brigham university in 1991[1]. The COX-2 gene (PTGS.2 gene, prostaglandin synthase-2) in humans has been identified on chromosome 1q25 [32].

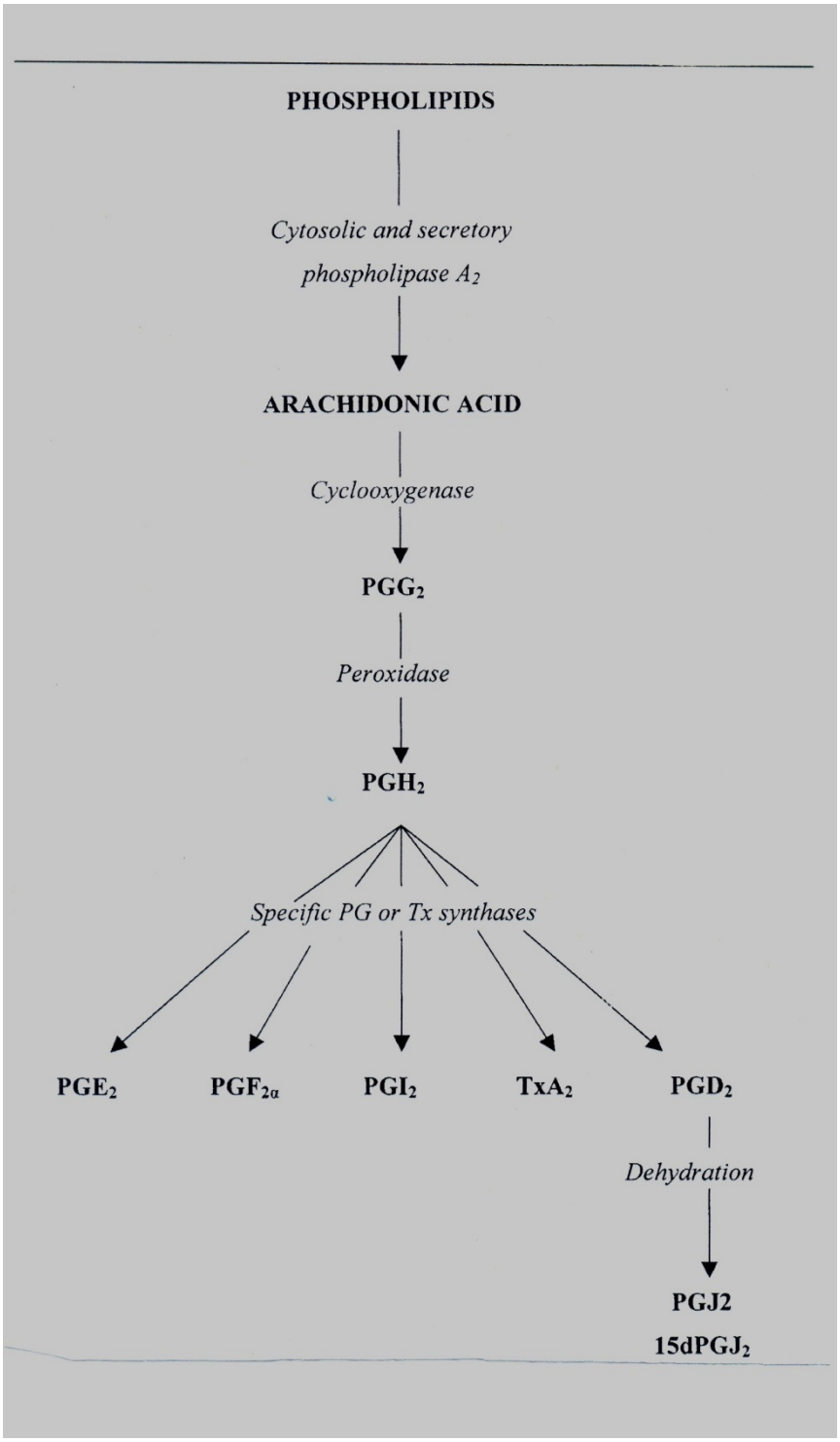


Fig 1 : shows the biosynthesis of prostaglandins[30]

Tissue distribution of cox-1 and cox-2:

COX-1 is the 'constitutive isoform' expressed in nearly all cell types under basal conditions. It has been found in blood vessels, interstitial cells, smooth muscle cells, platelets, mesothelial cells, stomach and kidney [33].

COX-2 isoform is not constitutive in most tissues except in placenta, macula densa of the kidney and brain [31]. COX-2 is however inducible in many cell types- synoviocytes, endothelial cells, chondrocytes and macrophages by cytokines like IL- 1 (Interleukin-1), Interleukin-2, Tumor Necrosis Factor-alpha and bacterial lipopolysaccharide (LPS) [34]. Cytokines such as interleukin-4, IL-10, IL-13 and corticosteroids decrease the induction of COX-2.

Functions of cox-1 and cox-2:

Stomach:

Cytoprotective prostaglandins eg: prostacyclins are synthesized by COX-1, although small quantities of COX-2 is expressed constitutively [35]. COX-1 promotes crypt stem cell survival and proliferation. It maintains the integrity of the mucosal epithelium by enhancing mucosal blood flow by vasodilation.

Kidney:

PGs are synthesized mainly by COX-1, low levels of COX-2 has been detected. PGs do not maintain normal renal blood flow, but are important in a compromised kidney and in patients with congestive heart failure, cirrhosis and renal insufficiency [29].

Platelet:

The only isoform detectable in the platelet is COX-1 which produces thromboxane. Thus prophylaxis against thromboembolic disease can be achieved by aspirin.

Gestation and parturition:

Both COX-1 and COX-2 are expressed in the uterine epithelium and are important for implantation of the ovum and in angiogenesis during placenta formation. COX-2 plays a role in inducing uterine contractions during labor. In addition both COX-1 and COX-2, the former at a higher level, is expressed in fetal hearts, kidneys, lungs, brain and the decidual lining of uterus [36].

CNS:

COX-1 is distributed in neurons throughout the brain, being most prevalent in forebrain. COX-2 expression is confined to cortex, hippocampus,

hypothalamus and spinal cord. In addition, COX-2 is also detected in the non-neuronal cells.

The major PGs of the CNS are PGE_2 and PGD_2 which are involved in modulation of autonomic nervous system and sensory processing [37]. PGE_2 generates signals that activate the thermoregulatory centre of the anterior hypothalamus thus playing a role in febrile response. The COX-2 induced in the endothelial cells of cerebral blood vessels is responsible for PGE2 release [38] .

In the spinal cord, the nociceptive process is brought about by COX-2 causing hyperalgesia.

Cox-2 in colon:

COX-2 expression in the normal colonic mucosa is either low or absent [39]. Low levels of COX-2 are derived from macrophages, vascular endothelial cells and neuroendocrine cells in the normal mucosa [39]. However it is an early response gene that is induced rapidly in response to growth factors, cytokines, oncogenes and phorbol esters [40].

The promoter region of COX-2 consist of many transcription factor binding sites such as nuclear factor Kb, nuclear factor of IL-6, AMP (Cyclic Adenosine Monophosphate) and hypoxia inducible factors (HIF-1) – all of which upregulate COX-2 [41] .

STRUCTURE OF CYCLOOXYGENASE:

COX-1 and COX-2 have a similar molecular weight of 70K.Da and are almost identical in length. The tertiary and quarternary structures are also identical (Fig 2A). Each subunit has three structural domains: a short N-terminal epidermal growth factor (EGF) domain, an alpha-helical membrane binding moiety and a C-terminal catalytic domain [42].

COX-1 and COX-2 are bifunctional enzymes that carry out two consecutive chemical reactions. Both the cyclooxygenase and the peroxidase active sites are located in the catalytic domain, which accounts for 80% of the protein.

Around 63% of their 600 aminoacids are in an identical sequence. Some substitutions are seen among the two namely- Isoleucine in COX-1 is exchanged for valine in COX-2 at positions 434 and 523 (Fig 2B).

Inspite of the structural identity, differences are seen in substrate and inhibitor selectivity. COX-2 acts on a wider range of fatty acids as substrate which include α-linolenic acid, linoleic acid than COX-1 [29] .

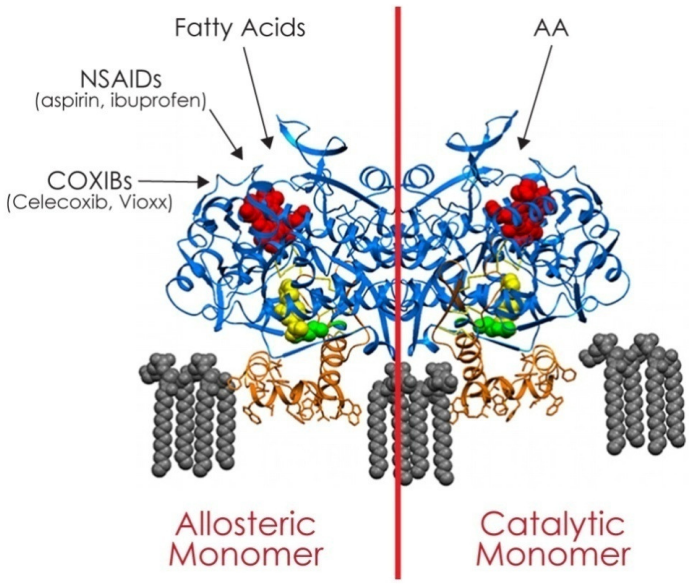


Fig 2A : Shows the overlapping structure of COX-1 and COX-2 [42]

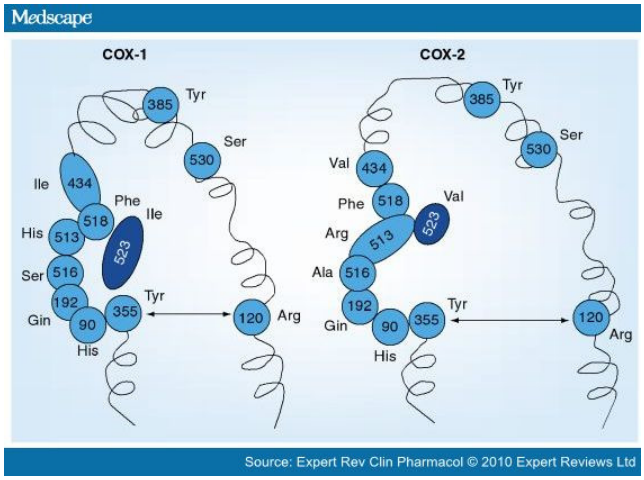


Fig2B : the structural variation between COX-1 and COX-2[42]

COX-2 AND INFLAMMATORY BOWEL DISEASE :

Crohn's disease characterized by chronic inflammation may occur at any level of the digestive tract. Mutations in the NOD2 / CARD 15 gene located on chromosome 16 is the predisposing event for the development and maintenance of the inflammatory process.

Overexpression of COX-2 has been seen in both the epithelial cells and inflammatory cells in response to the inflammatory cytokines [43]. Romero and others have found a significant association between COX-2 expression and epithelial alterations such as ulceration, mucin depletion, presence of paneth cells [43]. COX-2 overexpression was more frequently seen in the epithelial cells than in inflammatory cells. COX-2 immunostaining was demonstrated in normal mucosa in the control group of Romero's study in contrast to other studies which have reported the absence of the expression in normal epithelium [44].

As already reviewed, extensive ulcerative colitis of more than 8 years duration is an important risk factor for colonic epithelial dysplasia and adenocarcinoma[13] . Agoff and others have observed COX-2 expression in both the actively inflamed, non dysplastic mucosa and in non inflamed mucosa in all stages of neoplastic progression. COX-2 overexpression has been demonstrated in low -grade dysplasia, high-grade dysplasia and adenocarcinoma [45,46] . The

authors documented a more uniform diffuse cytoplasmic expression in all grades of dysplasia compared to adenocarcinoma where the COX-2 expression was not uniform with only 50% of cells staining in foci, although some foci had marked, uniform COX-2 overexpression [45] .

ROLE OF COX-2 IN CARCINOGENESIS:

COX-2 induction has been associated with various premalignant and malignant lesions of colon, lung, breast, prostate, bladder, stomach and esophagus [41]. COX2 and its major downstream product PGE_2 play an important role at multiple levels in colorectal carcinogenesis [47]. COX-2 is involved in activation and formation of carcinogens, inhibition of apoptosis, providing replicative potential, production of angiogenic factors and enhancing the metastatic potential[40].

Mechanism of overexpression of COX-2 in colorectal carcinoma :

Activating mutations in the PTGS2 gene, the gene encoding COX-2 in humans have not been reported but other mechanisms that cause COX-2 overexpression include the activation of WnT pathway, loss of APC gene and RAS-MAPK pathway via the growth factor receptors- EGFR, C-Met [47] (fig 3).

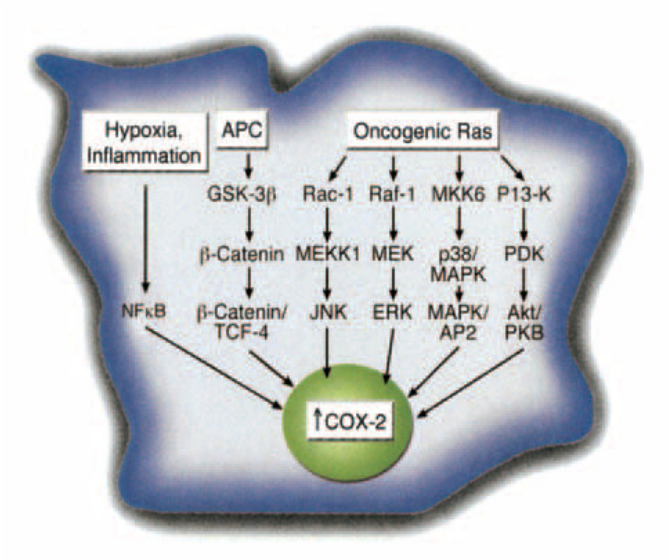


Fig3 : Shows COX-2 activation pathways [47]

Mechanism of cox-2 carcinogenesis [47] :

(i)Evasion of apoptosis

(ii) Providing self sufficiency of growth signals

(iii) Decreasing the sensitivity to antigrowth signals

(iv) Providing limitless replicative potential

(v) Sustaining angiogenesis

(vi) Tumor progression- Invasion and metastasis

(vii) Evasion of antitumor immune response.

(i)Evasion of apoptosis:

COX-2 increases the expression of bcl-2, antiapoptotic protein [47] . In addition it also increases the cell survival by activating the survival pathways such as PI3K /AKT (Phosphatidyl Inositol triphosphate / AKT), cAMP / protein kinase A signalling and PPAR (nuclear peroxisome proliferator – activator receptor – delta) pathways [47]. The inhibition of Fas- mediated apoptosis is another mechanism observed in cholangiocarcinoma [48]. Thus both the extrinsic and intrinsic pathways of apoptosis are inhibited.

The PPAR acts as a transcriptor factor, controlling the expression of genes involved in lipid metabolism. The dietary fat provides agonist ligands for PPARs. Thus COX-2 overexpression along with increased intake of dietry fat together play a role in carcinogenesis [49] .

The suppression of apoptosis by COX-2 also determines the susceptibility of tumor cells to radiotherapy and chemotherapy. COX-2 overexpression decreases the sensitivity of tumor cells to cytotoxic therapy [47] .

(ii) Providing self-sufficiency of growth signals :

As described above COX-2 / PGE2 activate PI3K / AKT, RAS-MAPK / ERK, EGFR signalling and cAMP / protein kinase A pathways by which tumor cells acquire growth autonomy even in the absence of activating mutations.

More important is the activation of the APC/β catenin pathway [47]. Mechanisms prevail within the tumor to maintain the overexpression of COX-2.

During hypoxia, the HIF-1 (Hypoxia- Inducible Factor-1) upregulates COX-2 expression which activates the RAS-MAPK pathway which acts in a positive feedback loop to maintain the COX-2 / PGE_2 [50].

(iii) Insensitivity to antigrowth signals :

Overexpression of COX-2 down regulates the TGFβ type II receptor (Transforming Growth Factor β) which normally blocks the cell progression through G1 phase of cell cycle by suppression of c-MYC and activation of cycle cycle inhibitors.

Another mechanism is to block the differentiation of crypt epithelial cells in the upper third of the crypt, a physiological process occurring in the normal crypt epithelium. The progenitor proliferating cells in the lower third of the crypt become differentiated and are shed into the lumen. This is blocked by COX-2 / PGE_2 by the activation of APC / β catenin pathway which maintain cells in a progenitor state. Few studies have shown that the COX-2 /PGE_2 can directly block the differentiation [47].

(iv) Limitless replicative potential :

The proliferative compartments in the intestinal crypts are maintained through activation of the WnT pathway. Perturbations of the APC /β catenin pathway have been detected in aberrant crypt foci, the earliest lesions of colorectal tumor.

Following the loss of APC gene, there is increased expression of COX-2. This further activates the activity of the already elevated levels of the β catenin following the loss of APC. The β catenin translocates to the nucleus and acts as a transcription factor along with TCF-4 (T-Cell Factor 4) complex. The β catenin – TCF4 complex binds to specific target genes – c MYC, cyclin D, PPARs, COX-2 and increase their levels thereby causing cell proliferation. On the other hand, by increasing the COX-2 levels, a positive loop is formed [41] (Fig 4,5). TCF binding sites have been identified in the promoter region of COX-2.

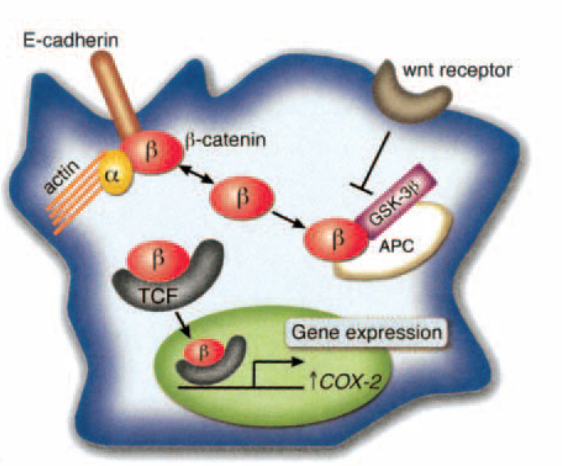


Fig 4 : shows COX-2 overexpression by free β catenin following loss of APC . COX-2 in turn stimulates β catenin forming a positive loop [41]

(v) Sustained angiogenesis :

COX-2 induces the production of angiogenic factors such as VEGF (Vascular Endothelial Growth Factor) and b-FGF (basic- Fibroblast Growth Factor). It also activates the integrinαVβ3 which is essential for endothelial survival, spreading and migration [51]. Wu and others in their study have found a strong correlation between COX-2 expression and VEGF expression both by immunohistochemistry and RT-PCR [52].

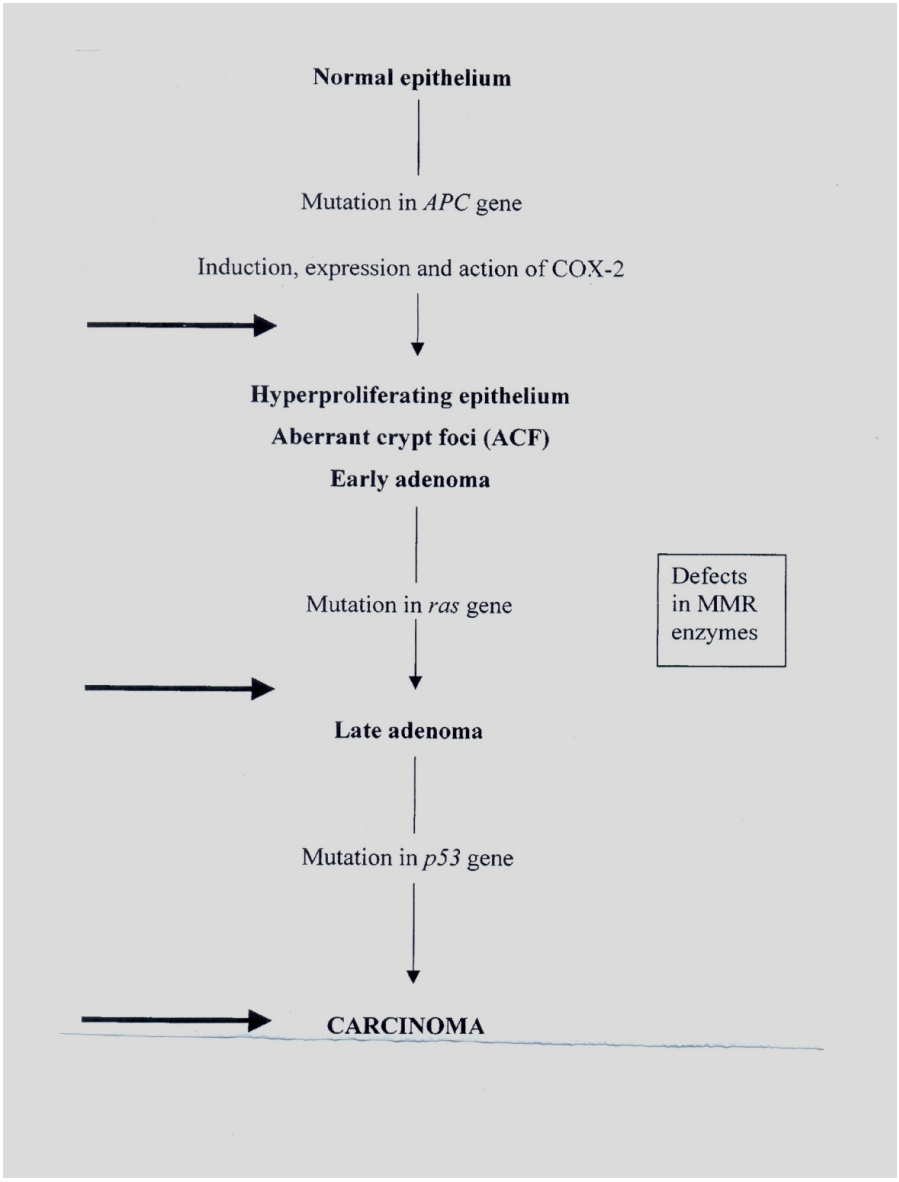


Fig 5 : shows colorectal carcinogenesis and action of COX-2 inhibitors . Loss of APC gene is followed by induction of COX-2 causing the progression of carcinoma. Defects in DNA mismatch repair genes(MMR) occur further during carcinogenesis .The heavy arrows show points at which inhibition of COX-2 will block progression.[30]

(vi) Tissue invasion and metastasis :

As COX-2 / PGE_2 levels increase, there is loss of E- cadherin and transactivation of Hepatocyte growth factor / c-Met signalling which contribute to tumor progression.

Loss of E cadherin is associated with elevation of vimentin leading to epithelial-mesenchymal transition (EMT) which makes the tumor cells more migratory [53]. HGF / c-Met increases β catenin and urokinase type plasminogen activator receptor expression which favours proliferation and invasion respectively[47]. A strong expression of MMP-2 (Matrix Metalloproteinase-2) has been identified in cells with COX-2 expression thereby increasing the invasiveness of the COX-2 expressing neoplastic cells by 6 times [54].

(vii) Evasion of antitumor immune response :

Cytotoxic CD8 T cells elicit antitumor response. PGE_2 shift the production of cytokines away from a TH1 cell profile thereby reducing the activation of CD8 T cells [55] .

In addition to these mechanisms, malondialdehyde, a by product of COX-2 mediated prostaglandin synthesis and lipid peroxidation is mutagenic and causes genetic damage [56].

COX-2 AND ITS RELATIONSHIP WITH TUMOR BIOLOGICAL CHARACTERISTICS:

Among the many prognostic factors reviewed earlier in this study, depth of invasion, stage of tumor, presence of lymph node metastasis, distant metastasis, vascular and neural invasion are the important factors in determining the prognosis. The relationship of COX-2 expression with each of these has been discussed below:

(i) Depth of invasion :

A significant association between COX-2 expression and the depth of invasion has been described by Soumaro LT and others in their study of 288 resected specimens of colorectal carcinoma [57]. COX-2 expression increased from 56.4% in T1 and T2 tumors to 74.1% and 96.3% in T3 and T4 tumors indicating the role of COX-2 in tumor progression.

A similar association has been seen by Wu AW and others in their study of colorectal carcinomas and adenomas. A strong COX-2 expression was seen in colorectal carcinoma, a moderate expression in adenoma and a weak to

absent expression in normal mucosa. Further a higher COX-2 positive T4 cases were seen compared to the T3 tumors [58] .

Comparing the COX-2 mRNA levels between the colorectal carcinoma and the normal mucosa by PCR method, it has been seen that COX-2 expression was significantly lower in the normal mucosa. In this study, Fugita and others further have demonstrated that COX-2 levels are significantly higher in tumors with deeper invasion [59] .

Other studies [60] however did not show an association since tumors were staged by Duke's system. The Dukes system does not definitely differentiate between different levels of invasion in the bowel wall [60].

(ii) ***Stage of tumor :***

Strong correlation between the stage of colorectal carcinoma and COX-2 expression has been documented by many series of studies in the literature. Sheehan KM and others found that patients with high COX-2 expression (grade 4 ie. expression in >50% tumor cells) were 4 times more likely to be classified as Dukes C and D than the patients with COX-2 expression in <20% of tumor cells [61]. Only the percentage of positive cells were taken into account in this study. The intensity of staining was not considered in the grading.

Similarly in another study, comparing the COX-2 expression with microvessel density, Masunaga R and others have observed a strong association

between COX-2 expression and Dukes stage B, C and D [62]. Both the percentage of cells and the staining intensity were included in assessing the COX-2 expression in this study.

A similar correlation was noted in two other studies by Al-Maghrabi [63] and Soumaro [59] where the tumors were staged by AJCC system (American Joint Committee Cancer staging system). In the former higher COX-2 expression was seen in stage III / IV tumor compared to stage I /II, while in the later a gradual increase was observed from stage I to stage IV.

(iii) ***Lymph node metastasis:***

Patients with high COX-2 expression were 4 times more likely to have lymph node metastasis [61] . This finding by Sheehan K M and others has been supported by Al-Maghrabi who noted that 63% of tumors with lymph node involvement tested positive for COX-2 while only 37% of the cases with no COX-2 expression had lymph node involvement [63].

In addition to confirming these observations, and furthur evaluating the association between COX-2 expression and lymph node involvement, Masunga and others found a significant correlation between increased COX-2 expression and presence of more than three metastatic lymph nodes (N2) [62]. A similar further evaluation revealed more frequent COX-2 expression in metastatic lymph nodes compared to the primary tumors [57,63].

(iv)Distant metastasis:

Among the three studies evaluating the association of COX-2 overexpression with distant metastasis, Soumaro LT [57] and Al-Maghrabi [63] in their works have found a higher hematogenous metastasis in COX-2 positive patients. The former in addition have described 100% COX-2 expression in the metastatic sites compared to a 71% expression in the primary tumor.

Tomozawa S and others further classified the COX-2 positive tumors into low positive and high positive groups considering both the intensity and extent of positive reaction of tumor cells [64]. Low positive groups included grade 1 and grade 2 which showed weaker COX-2 expression than mononuclear cells. The high positive groups included grade 3 and 4 which showed COX-2 expression either similar or stronger than mononuclear cells. The high COX-2 expression strongly correlated with hematogenous metastasis.

In all the three studies COX-2 overexpression was in addition also associated with tumor recurrence.

(v) Microscopic grade and type:

Increased COX-2 expression is significantly correlated with tumor differentiation. Poorly differentiated carcinomas more frequently expressed COX-2 compared to the well and moderately differentiated carcinoma [62,63] .

However no such statistically significant association was observed by Soumaro LT and Tomozawa S in their studies.

Evaluating the COX-2 expression in carcinoma with signet ring cell and mucinous component, Ogino S and others observed that the mucinous group showed higher levels of COX-2 along with KRAS mutations than the signet ring group which showed lower COX-2 level [65]. However an elevated COX-2 expression was noted both in the mucinous and signet ring cell colorectal carcinoma by Baba and others who found in addition, $PTGER_2$ overexpression to be associated with microsatellite instability [66] .

(vi) Tumor size :

Fujita T and others have demonstrated high COX-2 mRNA levels in larger tumors. The tumors were divided into three classes: ≤ 3 cm, ≤ 6 cm and > 6 cms and the tumor surface areas were calculated. The COX-2 mRNA levels were then determined by PCR method [59].

Other studies also showed greater COX-2 expression in larger tumors, where COX-2 expression was determined by immunohistochemistry [57,61,62] . A maximum tumor diameter of > 3 cms was regarded as an unfavourable findings by Masunaga R and others [62] . No such association was seen in other studies by Tomozawa S [64] .

(vii) Tumor location :

A more frequent COX-2 expression in left colonic carcinoma (67%) compared to right colonic carcinoma (33%) has been reported by Nasir A and others [67] . The authors give a possible explanation for the same relating it to the genetic alterations – APC mutations seen frequently in the left sided carcinoma. In addition, reduced COX-2 expression has been documented in DNA mismatch repair gene defective tumors [68,69] .

However no such association between location of tumor and COX-2 expression has been reported by others [57,63].

(viii) Vascular, lymphatic and neural invasion :

More frequent vascular and lymphatic invasion has been reported in tumors with COX-2 overexpression by Soumaro LT [57]. However no such significant association was seen by Sheehan and others who evaluated the relation between neural invasion in addition to vascular and lymphatic invasion with COX-2 expression [61] .

COX-2 : A TARGET FOR CANCER CHEMOTHERAPY

It can be inferred from literature review that colorectal carcinoma with higher COX-2 gene expression grow larger and in a more invasive manner. Further a significant correlation between high COX-2 expression and a shortened patient survival has been documented [57,61,63] . Sheehan KM and others found a 5 years survival rate of 91.6% in the absence of COX-2 compared to 40.5% in tumors expressing COX-2 [61].

Taking into consideration both the stage of the tumor and COX-2 expression, Soumaro LT and others have reported a five year survival rate of COX-2 negative and COX-2 positive cases in stage I and II as 97% and 82% respectively and that in stage III as 88% and 67% [57] . Analysing the morbidity of the patients, a major difference was seen in the disease free survival between patients with COX-2 positive and negative tumors [63]. A recurrence of 85% was seen in patients with COX-2 expressing tumors compared to 40% in patients with COX-2 negative tumors.

Colorectal tumors with COX-2 overexpression are at high risk for local, distant recurrence and because of the strong adverse impact on survival, these patients are appropriate candidates for COX-2 inhibitor therapy.

Epidemiological studies show that the regular use of aspirin and other NSAIDS reduces the risk of colorectal carcinoma by 40-50%. Further a

regression of preexisting adenomas in patients with FAP (Familial Adenomatous Polyposis) has been documented [70] .

However the use of aspirin is limited by its adverse effects on the gastric mucosa since both the activity of COX-1 and COX-2 are blocked thereby decreasing the levels of cytoprotective prostaglandins formed by COX-1 activity. This necessitates the need for a COX-2 selective inhibitor.

Selective COX-2 inhibitors include- Celecoxib, Rofecoxib, L-745,337, SC 58125, Etodolac and Meloxicam . The newer drugs L-745,337 and SC 58125 have 100 fold selectivity for COX-2[71]. Another COX-2 selective inhibitor NS-398 has been found to induce apoptosis by elevating caspase-3 activity.

Celecoxib inhibited the incidence and multiplicity of colon tumors by 93 % and 97%. Rofecoxib enhances the effects of antineoplastic agents like 5-Flurouracil [70] . The drug decrease the expression of COX-2 along with cyclin D1, cytosolic β catenin, MMP-2 and MMP-9, VEGF as seen by animal studies .

In addition to chemotherapy, prognosis after radiotherapy is also associated with COX-2 levels as determined by Heer PD and others . The authors observed that tumors with high COX-2 expression after radiotherapy showed higher rate of distant recurrences, poor disease-free survival and poor overall survival [69] . This is because COX-2 is known to induce bcl-2 and cause

apoptosis resistance as already discussed. Addition of COX-2 inhibitors to preoperative radiotherapy therefore may help to reduce metastasis and improve survival. Celecoxib has been evaluated for use in combination with chemotherapy for treating patients with metastatic disease who failed prior chemotherapy.

COX-2 AND OTHER CARCINOMA :

COX-2 overexpression is not confined to only colorectal carcinoma. An overexpression has been seen in lung [72] , breast [73] , prostate [74] ,esophagus [75] and endothelium [47]. COX-2 overexpression has been seen in the non-small cell lung cancers- adenocarcinomas and squamous cell carcinomas [72] .

In patients with prostatic carcinoma with metastasis, a strong COX-2 expression along with Ki-67 expression was noted in the epithelial cells compared to the non-metastatic prostatic cancer group [74] . Further high intensity of COX-2 staining tumor cells was strongly associated with prostate cancer related death.

Marked COX-2 expression has been documented in squamous cell carcinoma and squamous dysplasia with no expression in normal epithelium in the esophagus by RT-PCR [75] .

Analysing the effect of COX-2 overexpression in endometrial carcinoma, Ohnos and others found that its overexpression was associated with advanced FIGO stage and invasion into outer half of the myometrium [76]. However grade of differentiation, menopausal status and lymph node metastasis had no association with COX-2 overexpression.

Materials and Methods

MATERIAL AND METHODS :

65 consecutive cases of colorectal carcinoma between January 2009 to December 2013 were retrieved from the records of the pathology department at PSG IMS&R. The paraffin- embedded H & E slides of the 65 cases were examined. The location, histological type and grade of the neoplasm, depth of invasion, lymph node metastasis were observed. Clinical details which included the age, sex, stage of tumor were recorded. The representative slides were selected in each case which included – primary tumor, lymph node, satellite nodule (if any), distant metastatic deposits (if any). The cases which was diagnosed as medullary carcinoma, neuroendocrine carcinoma, squamous carcinoma, adenosquamous carcinoma and choriocarcinoma were excluded. Immunohistochemical staining for COX-2 was done by the following procedure [77].

Section were cut at approximately 4 micrometer, floated on to Poly-L-Lysine coated slides and incubated at 37 degree Celsius for one day and further incubated at 58 degree Celsius over night. Deparaffinization was done in 2 changes of Xylene each for 15 min followed by dexylinization in 2 changes of absolute alcohol. Dealcoholisation was done by graded alcohol 90% and 70 % alcohol each for 1 min. Rehydration was done in tap water for 10 min and rinsed in distilled water for 5 min.

Antigen retrieval was done by pressure cooking in citrate buffer (pH - 6.0) for 10 min and leaving the pressure cooker in the sink with water for 20 min . The slides were then rinsed in distilled water for 5 min . The slides were transferred to TBS (Tris-Buffer Solution) (pH 7.6) for 5 min.

Peroxidise block was done for 10 to 15 min. The slides were then washed in TBS buffer 3 times each lasting for 5 min. To block non specific reaction with the other tissue antigen, power block was done for 15 min. The slides were drained and the sections were covered with concerned primary antibody to detect the tumor markers by antigen antibody reaction for 1 hr. The slides were then washed in TBS 3 times, each for 5 min to wash the unbound antibodies.To enhance the reaction between primary and secondary antibodies, super enhancer was added and left for 30 min. It was washed with TBS buffer 3 times, each for 5 min to remove the unbound antibodies. To elongate chain and also to label the enzyme, Super sensitive Poly- HRP was added. This was followed by washing in TBS buffer to remove the unbound antibodies. To give colour to the antigens, the slides were treated with colour development solution for 5-8 min. It was followed by wash in TBS buffer and the tap water each for 5 min. Counterstaining with haematoxylin stain for 1 min was done and the excess stain was washed in tap water for 5 min. The slide is air dried, cleared in xylene and mounted with DPX.

A section of lung adenocarcinoma was used as positive control and the negative control was the same tissue incubated without secondary antibody[70].The Antibody solutions used were THERMO Scientific- Monoclonal Rabbit Anti-Human COX-2 clone . The chromogen in the colour development solution was 3'3' diaminobenzidine (DAB).

COX-2 cytoplasmic staining was evaluated using a method which takes into consideration both the proportion of positive cells and the intensity of staining [57,64] . The method is as follows:

EXTENT OF STAINING (proportion score PS)	**PERCENTAGE OF POSITIVE CELLS**
0	0%
1	1% - 25%
2	26% - 50%
3	51% - 75%
4	76% - 100%

INTENSITY SCORE (IS)	INTENSITY OF POSITIVITY
0	Negative
1	Weak *
2	Intermediate **
3	Strong ***

*Weaker than inflammatory cells

**Same as inflammatory cells

***Stronger than inflammatory cells

The extent of staining and the intensity of staining were added together for a total score.

Total score (PS + IS)	Interpretation
0 , 2	Negative
3 , 4 ,	Low positive
5 , 6 , 7	High positive

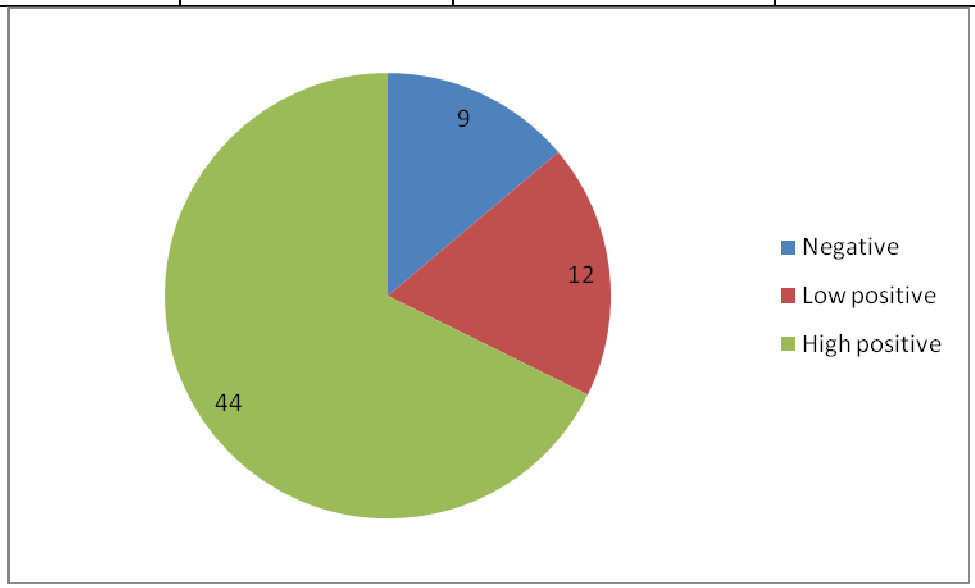
Results

RESULTS :

Of the 65 cases of colorectal carcinomas included in the study, 56 (86.2%) cases expressed COX-2 while 9 cases (13.8%) were COX-2 negative. Twelve of the 56 positive cases (21.4%) (12/65 = 28.5%) had a total COX-2 score of either 3 or 4 and were classified as low positive. The remaining 44 cases (78.6%) (44/65 = 67.7%) were high positive with a COX-2 score between 5 and 7 as shown in Table 1.

Table 1 : Shows the distribution of COX-2 expression in colorectal carcinoma :

TOTAL NO. OF CASES	**COX-2 EXPRESSION**		
	NEGATIVE	**POSITIVE**	
		LOW POSITIVE	**HIGH POSITIVE**
65	9 (13.8%)	12 (28.5%)	44 (67.7 %)



COX-2 NEGATIVE CASES :

4 of the 9 COX-2 negative cases did not show any expression of COX-2. Less than 25% of tumor cells of 5 other cases however weakly expressed COX-2 accounting to a total score of 2 which was considered negative. The details of the 9 COX-2 negative cases have been shown in table-2.

No lymph node metastasis was seen in 7 of the nine (77.8%) COX-2 negative cases. Only 2 cases (22.2%) had tumor in the lymph node and the tumor cells in one of them were low positive for COX-2.

7 cases (77.8%) and 2 cases (22.2%) were in stage II and stage III respectively. There were no stage I and stage IV tumors. 3 cases (33.3%) were well-differentiated while 6 (66.7%) were moderately-differentiated carcinoma.

Table 2 : Shows Stage, Microscopic grade, COX-2 score and Location of COX-2 negative Carcinoma:

Sl.no	STAGE OF TUMOR	MICROSCOPIC GRADE OF ADENOCARCINOMA	COX-2 SCORE	LOCATION OF TUMOR
1	II A T3N0M0	Well-differentiated	0	Left
2	II B T4N0M0	Moderately differentiated	0	Left
3	II A T3N0M0	Moderately differentiated	2	Right
4	IIIB T3N1M0	Moderately differentiated	2	Left
5	IIB T4N0M0	Moderately differentiated	2	Right
6	IIB T4N0M0	Moderately differentiated	0	Right
7	IIA T3N0M0	Moderately differentiated	0	Right
8	IIA T3N0M0	Well differentiated	2	Right
9	III T1N1M0	Well differentiated	2	Left

T1, T3 and T4 tumors constituted 11.1% (1 case), 55.6% (5 cases) and 33.3% (3 cases). However the satellite nodules of the T4 tumors expressed COX-2 (low positive).

COX-2 LOW POSITIVE CASES :

The stage and the degree of differentiation of the 12 low positive cases (12 of 65, 18.5%) has been shown in Table- 3.

Nine cases (75%) had no evidence of tumor in the lymph node. Of the 3 cases (25%) with lymph node deposits, a similar COX-2 expression was seen in both the primary tumor and the lymph node deposits in 2 cases while a higher expression was seen in the lymph node deposits in the third case in the N2 stage.

Two stage I tumors (16.7%), seven stage II tumors (58.3%) and three stage III tumors showed low positivity. Histological examination revealed 4 (33.3%) well differentiated, 8 (66.7%) moderately differentiated, 2 (16.7%) T2 and 10 (83.3%) T3 carcinoma. No T4 or T1 carcinoma was seen in this category.

TABLE-3 : Shows Location , Stage, Microscopic grade, COX-2 score of COX-2 Low positive carcinoma :

Sl.no	LOCATION OF TUMOR	STAGE OF TUMOR	MICROSCOPIC GRADE OF ADENOCARCINOMA	COX-2 SCORE
1	Left	II T3N0M0	Well differentiated	4
2	Right	IIIB T3N1M0	Well differentiated	4
3	Left	IIA T3N0M0	Moderately differentiated	4
4	Left	IIIB T3N1M0	Moderately differentiated	4
5	Left	IIA T3N0M0	Moderately differentiated	3
6	Left	IIA T3N0M0	Moderately differentiated	3
7	Left	IIA T3N0M0	Moderately differentiated	3
8	Left	IIA T3N0M0	Moderately differentiated	3
9	Left	IIA T3N0M0	Moderately differentiated	4
10	Right	IIIC T3N2M0	Moderately differentiated	4
11	Right	I T2N0M0	Well differentiated	4
12	Left	I T2N0M0	Well differentiated	4

COX-2 HIGH POSITIVE CASES :

The stage and degree of differentiation of the 44 (44/65 = 67.7%) tumors expressing high COX-2 has been shown in Table-4. Lymph node deposits was seen in 21 cases (47.7%) of which 11 were in N1 stage and 10 were in N2 stage. In all these cases both the primary tumor and lymph node deposit showed a high intensity of COX-2 expression except in 2 cases where the primary tumor showed a high positive COX-2 expression, but the lymph node showed a lower COX-2 expression. 23 cases (52.3%) did not have lymph node metastasis.

One (2.2%) T2 tumor, 34 (77.3%) T3 tumors and 9 (20.5%) T4 tumors were present in this group. There was no T1 tumor. Stage I, stage II, stage III and stage IV tumor constituted 1 case (2.2%), 20 cases (45.5%), 20 cases (45.5%) and 3 cases (6.8%) respectively. The organs involved by distant metastasis in the three stage IV tumors were fallopian tube and ovary in two of them and an umbilical nodule in the third. One of the former tumors had in addition spread to the uterus. Nine cases (20.6%), 28 cases (63.6%) and 5 cases (11.4%) were well differentiated, moderately differentiated and mucin secreting adenocarcinoma. In addition, there were 1 case each (2.2%) of poorly differentiated and signet ring cell carcinoma.

TABLE -4 : Showing Location, Stage, Microscopic grade and COX-2 score in COX-2 high positive carcinoma :

Sl.no	LOCATION OF TUMOR	STAGE OF TUMOR	MICROSCOPIC GRADE AND TYPE OF ADENOCARCINOMA	COX-2 SCORE
1	Left	IIA T3N0M0	Moderately differntiated	7
2	Left	IIIC T3N2M0	Moderately differentiated	7
3	Right	IIA T3N0M0	Moderately differentiated	6
4	Left	IIA T3N0M0	Moderately differentiated	5
5	Right	IIIB T3N1M0	Mucinous adenocarcinoma	6
6	Left	IIA T3N0M0	Moderately differentiated	5
7	Right	IIA T3N0M0	Mucinous adenocarcinoma	6
8	Right	IIIB T4N1M0	Poorly differentiated	7
9	Transverse	I T2N0M0	Mucinous adenocarcinoma	7
10	Left	IIIC T3N2M0	Well differentiated	6
11	Left	IIIB T3N1M0	Well differentiated	6
12	Left	IIA T3N0M0	Well differentiated	6
13	Right	IIIB T3N1M0	Moderately differentiated	7
14	Left	IIB T4N0M0	Moderately differentiated	5
15	Left	IIA T3N0M0	Moderately differentiated	7

Sl.no	LOCATION OF TUMOR	STAGE OF TUMOR	MICROSCOPIC GRADE AND TYPE OF ADENOCARCINOMA	COX-2 SCORE
16	Transverse	IIA T3N0M0	Moderately differentiated	6
17	Left	IIIC T4N2M0	Mucinous adenocarcinoma	6
18	Right	IIIB T3N1M0	Signet ring cell carcinoma	7
19	Left	IIIB T4N1M0	Moderately differentiated	7
20	Left	IIA T3N0M0	Moderately differentiated	5
21	Right	IIB T4N0M0	Well differentiated	7
22	Left	IIA T3N0M0	Well differentiated	7
23	Tranverse	IIA T3N0M0	Well differentiated	5
24	Left	IIIB T3N1M0	Moderately differentiated	7
25	Left	IIIC T3N2M0	Moderately differentiated	6
26	Left	IV T4N0M1	Moderately differentiated	6
27	Left	IIIB T4N1M0	Moderately differentiated	6
28	Left	IIIC T3N2M0	Moderately differentiated	5
29	Left	IV T4N0M1	Moderately differentiated	6
30	Right	IIA T3N0M0	Moderately differentiated	6
31	Right	IIA T3N0M0	Moderately differentiated	6
32	Left	IIIC T3N2M0	Moderately differentiated	5

33	Right	IV T3N2M1	Moderately differentiated	7
34	Left	IIIB T3N1M0	Moderately differentiated	6
35	Left	IIA T3N0M0	Moderately differentiated	5
36	Right	IIA T3N0M0	Well differentiated	5
37	Left	IIIC T3N2M0	Moderately differentiated	7
38	Left	IIIC T3N2M0	Well differentiated	5
39	Right	IIIB T4N1M0	Mucinous adenocarcinoma	7
40	Left	IIIB T3N1M0	Well differentiated	6
41	Left	IIA T3N0M0	Moderately differentiated	7
42	Left	IIIC T3N2M0	Moderately differentiated	5
43	Right	IIA T3N0M0	Moderately differentiated	6
44	Right	IIA T3N0M0	Moderately differentiated	6

COX-2 EXPRESSION AND STAGE OF TUMOR :

There were 3 (4.6%), 34 (52.3%), 25 (38.5%) and 3(4.6%) tumors of stage I, II, III and IV respectively. The pattern of COX-2 expression has been shown in Table-5. Of the three stage I tumors, 2 (66.7%) were COX-2 low positive and 1 (33.3%) was COX-2 high positive. None of stage I tumors were COX-2 negative. Seven (20.6%) COX-2 low positive, 20 (58.8%) COX-2 high positive and 7(20.6%) COX-2 negative tumors constituted the thirty four stage

II tumors. The 25 stage III tumors included 3 (12%), 20 (80%) and 2 (8%) COX-2 low positive, high positive and COX-2 negative cases respectively.

TABLE-5: Showing relation between COX-2 status and the Stages of colorectal Carcinoma :

STAGE OF TUMOR	**COX-2 EXPRESSION**		
	NEGATIVE	**POSITIVE**	
		LOW POSITIVE	**HIGH POSITIVE**
Stage I (n=3)	Nil (0%)	2 (66.7%)	1 (33.3%)
Stage II (n= 34)	7 (20.6%)	7 (20.6%)	20 (58.8%)
Stage III (n = 25)	2 (8%)	3 (12%)	20 (80%)
Stage IV (n= 3)	Nil (0%)	Nil (0%)	3 (100%)

All the 3 stage IV tumors (100%) expressed strong COX-2 staining in over 70% of their tumor cells; all three were considered high positive. In addition, the primary tumor and the distant metastasis exhibited high COX-2 score in all the three cases. The lymph node deposits and a satellite nodule seen in two of these cases also had a COX-2 score of 7 (Table-6).

TABLE-6 : Shows COX-2 staining in stage IV carcinoma:

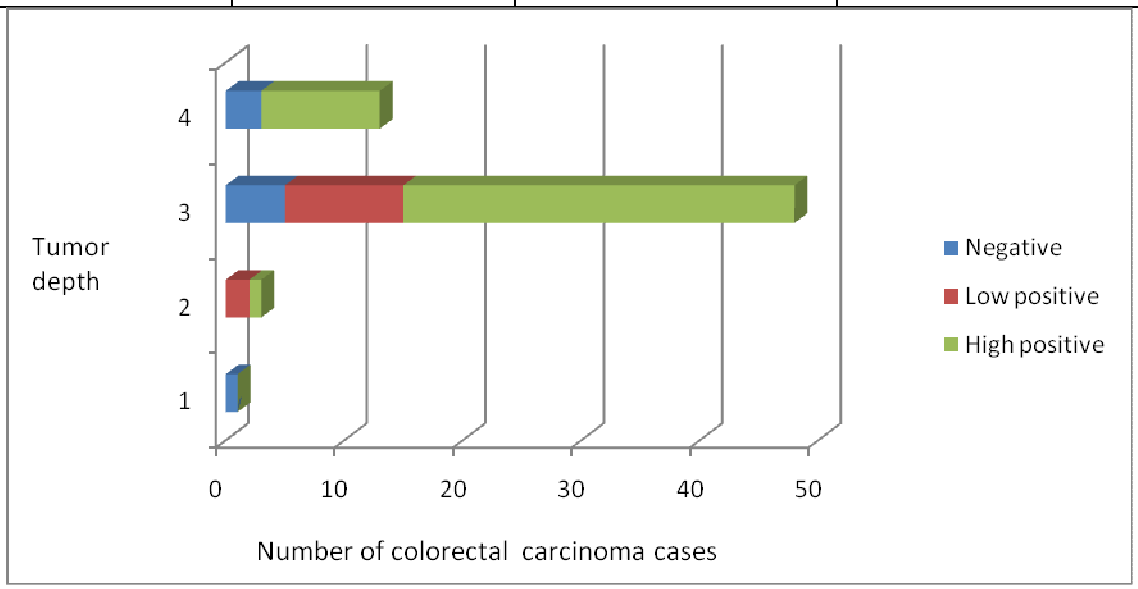
Sl. no	STAGE OF TUMOR	ORGAN OF METASTASIS	INTENSITY OF COX-2 STAINING (COX-2 SCORE)			
			PRIMARY TUMOR	LYMPH NODE	SATELLITE NODULE	METASTATIC SITE
1	T4N0M1	Fallopian tube & ovary	6	-	7	7 7
2	T4N0M1	Uterus, fallopian tube & ovary	6	-	6	5 6 5
3	T4N2M1	Umbilical nodule	7	7	7	7

<u>COX-2 AND DEPTH OF INVASION :</u>

Of the 65 cases of colorectal carcinoma, 1(1.5%), 3(4.6%), 48 (73.9%) and 13 (20%) were T1,T2, T3 and T4 tumors respectively. The intensity of COX-2 staining in these tumors have been shown in table-7.

TABLE-7 : Shows relation between COX-2 expression and depth of invasion of Colorectal carcinoma :

DEPTH OF INVASION	COX-2 EXPRESSION		
	NEGATIVE	POSITIVE	
		LOW POSITIVE	HIGH POSITIVE
T1 (n=1)	1 (100%)	Nil (0%)	Nil (0%)
T2 (n=3)	Nil	2 (66.7%)	1 (33.3%)
T3(n=48)	5 (10.4%)	10 (20.8%)	33 (68.8%)
T4 (n=13)	3 (23%)	Nil (0%)	10 (77%)



The single T1 tumor was COX-2 negative (100%). Of the 3 tumors in T2 stage, 2 (66.7%) were low positive while the other was high positive. Five COX-2 negative (10.4%), 10 low positive (20.8%) and 33 high positive(68.8%)

cases consitituted the 48 T3 tumors. The thirteen T4 tumors included 3 cox-2 negative (23%) and ten (77%) high positive tumors. None of T4 tumors were low positive for COX-2.

In addition, of the five COX-2 negative T3 tumors, three were Rt colonic carcinoma and 2 were from the Lt colon. Similarly the three COX-2 negative T4 tumors included 2 from the Rt colon and one from the Lt colon.

COX-2 EXPRESSION AND HISTOPATHOLOGICAL GRADE, TYPE:

The 65 cases of colorectal carcinomas were comprised of 16 cases (24.6%), 42 cases (64.7%) and 5 cases (7.7%) of well-differentiated, moderately differentiated and mucinous adenocarcinoma respectively. One case each (1.5%) of poorly differentiated and signet ring cell adenocarcinoma were present in the study. The pattern of COX-2 expression in these categories have been shown in Table-8.

Well-differentiated adenocarcinoma :

Of the sixteen well –differentiated adenocarcinomas, 3 (18.8%) COX-2 negative, 4 (25%) COX-2 low positive and 9(56.2%) COX-2 high positive were present (Table-8). Lymph node metastasis was seen in one of the four low

positive tumors and the tumor deposits in the lymph node were also COX-2 low positive. Of the nine high positive cases four had evidence of tumor in the lymph node which was also high positive. Both the primary tumor and lymph node showed 80% of tumor cells with moderate to strong COX-2 staining. One of the three COX-2 negative tumors had lymph node metastasis. In this case, about 10% of cells of the primary tumor exhibited a weak COX-2 expression giving a total score of 2 and was regarded as negative, while the tumor cells within the lymph nodes were low positive with a total score of 4.

TABLE-8 : Shows COX-2 expression in the different histological types and

Grade of colorectal carcinoma :

HISTOLOGICAL GRADE AND TYPE	**COX-2 EXPRESSION**		
	NEGATIVE	**LOW POSITIVE**	**HIGH POSITIVE**
Well differentiated adenocarcinoma (n=16)	3 (18.8%)	4 (25%)	9 (56.2%)
Moderately differentiated adenocarcinoma (n=42)	6 (14.3%)	8(19%)	28 (66.6%)
Poorly differentiated adenocarcinoma (n=1)	0	0	1(100%)
Mucinous adenocarcinoma (n=5)	0	0	5 (100%)
Signet ring cell adenocarcinoma (n=1)	0	0	1 (100%)

Moderately differentiated adenocarcinoma :

6 cases (14.3%) of COX-2 negative, 8 (19%) cases of low positive and 28 (66.6%) cases of high positive moderately differentiated adenocarcinoma were observed in the present study (Table-8).

One of the six COX-2 negative cases showed lymph node metastasis which was also COX-2 negative. Evidence of tumor in the lymph node was seen in two of the six low-positive cases. One of these had tumor in more than three lymph nodes (N2 stage) and a high COX-2 score was seen in the lymph nodes, while the primary tumor was low positive. The lymph node deposits in the other low positive tumor had a similar low COX-2 score as the primary tumor.

Of the twenty eight high COX-2 expressing moderately differentiated adenocarcinomas, twelve had tumor deposits in the lymph node with seven in N2 stage. In five of these tumors in N2 stage, a high COX-2 expression was seen both in the primary tumor and lymph node, while in two of them the primary tumor had a high COX-2 expression compared to lymph node deposits which were low COX-2 positive. A high COX-2 expression was seen both in the primary tumor and lymph node deposits in four of the five cases in N1 stage.

Poorly-differentiated adenocarcinoma :

The single case (1.5%) was COX-2 high positive, stage III (T4N1M0) tumor with total COX-2 score of 7 in the primary tumor, lymph node and satellite nodule.

Mucin secreting adenocarcinoma :

All the 5 cases expressed a high COX-2 score. Three of these tumors belonged to stage III and the other two were in stage I and stage II. The lymph node deposits in three stage III tumors and the satellite nodule in the two T4 tumors also were COX-2 high positive. These features have been summarised in table 9.

Signet ring cell adenocarcinoma :

The single case of signet ring cell adenocarcinoma present in the study was a stage IIIB tumor (T3N1M0) with a high COX-2 expression in the primary tumor and omental nodule and a low COX-2 score of 3 in the lymph node.

TABLE-9 : Shows Location, Stage and COX-2 expression in mucinous adenocarcinoma :

Sl.no	LOCATION	STAGE	COX-2 SCORE		
			PRIMARY TUMOR	LYMPH NODE	SATELLITE NODULE
1	Right	IIIB T3N1M0	6	7	No nodule
2	Right	IIA T3N0M0	6	No lymph node	No nodule
3	Transverse	I T2N0M0	7	No lymph node	No nodule
4	Left	III T4N2M0	6	7	7
5	Right	IIIB T4N1M0	7	7	7

Thus 81.25% of well differentiated adenocarcinoma expressed COX-2 while 85.7% of moderately differentiated carcinoma expressed in COX-2. All the cases (100%) of poorly differentiated, mucinous and signet ring cell adenocarcinoma expressed COX-2 (Table-8).

The relation of COX-2 expression with tumor stage, depth of invasion, microscopic type, grade and lymph node metastasis are summarised in Table 10.

Table 10:Shows COX-2 expression in various TNM stages, depth of invasion, microscopic grade and lymphnode metastasis.

COX 2 EXPRESSION	AJCC (TNM STAGING)				DEPTH OF INVASION				MICROSCOPIC GRADE					LYMPH NODE STATUS	
	I	II	III	IV	T1	T2	T3	T4	WELL DIFFERENTIATED	MOD DIFFERENTIATED	POORLY DIFFERENTIATED	MUCINOUS CARCINOMA	SIGNET RING CARCINOMA	No METASTASIS	METASTASIS
	3	34	25	3	1	3	48	13	16)	(42)	(1)	(5)	(1)	(39)	26
NEG(9)	0	7	2	0	1	0	5	3	3	6	0	0	0	7	2
LP (12)	2	7	3	0	0	2	10	0	4	8	0	0	0	9	3
HP (44)	1	20	20	3	0	1	33	10	9	28	1	5	1	23	21

COX-2 EXPRESSION AND LOCATION OF TUMOR :

Twenty two cases (33.9%) of Rt colon carcinoma, 40 cases (61.5%) of Lt colon carcinoma and 3 cases (4.6%) of transverse colon carcinoma constituted the 65 cases. The varying COX-2 expression has been shown in table 11

TABLE-11 : Shows cox-2 expression and tumor location :

LOCATION OF TUMOR	COX-2 NEGATIVE	COX-2 POSITIVE
Right colon (n=22)	5 (22.7%)	17 (77.3%)
Left colon (n= 40)	4 (10%)	36 (90%)
Transverse (n=3)	Nil	3 (100%)

Of the 22 Rt colonic carcinoma, 17 (77.3%) expressed COX-2 and 5 (22.7%) were negative. The forty cases of Lt colonic carcinoma included 4 (10%) COX-2 negative tumors and 36 (90%) COX-2 positive tumors.

COX-2 EXPRESSION AND SIZE OF TUMOR :

The greatest diameter of the nine COX-2 negative tumors varied between 2.5cm and 6.0 cms (average 4.6 cms) while those of 65 COX-2 positive tumors ranged from 2.5cms to 12.0 cms (average 5.3 cms).

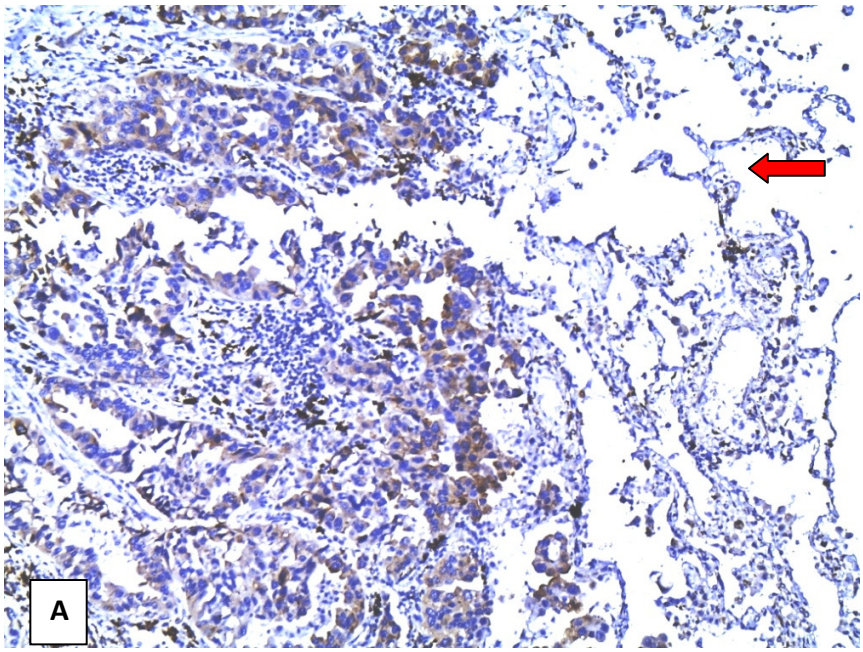
COMPARISON OF INTENSITY OF COX-2 STAINING BETWEEN PRIMARY TUMOR AND LYMPH NODE DEPOSITS IN STAGE III TUMORS :

Of the twenty five cases of stage III tumors, a similar COX-2 expression was seen both in the primary tumor and lymph node deposits in 19 cases (76%). In four cases (16%) a high COX-2 expression was seen in primary tumor while the lymph node deposits showed a low COX-2 score, however they were positive(shown by astrix in table 12). In one case (4%), both the primary tumor and lymph node deposit were negative and in one case (4%) the primary tumor was low COX-2 positive while the lymph node was high positive. These findings have been shown in table -12.

TABLE-12 : Shows COX-2 score in primary tumor and lymph node metastasis in stage III tumors

Sl.no	**TNM STAGE**	**COX-2 EXPRESSION**	
		PRIMARY TUMOR	**LYMPH NODE**
1	T3N2M0	7	7
2	T3N1M0	6	7
3	T4N1M0	7	7
4	T3N2M0	6	7
5	T3N1M0	6	6

6	T3N1M0	7	7
7	T3N1M0	7	3
8	T4N2M0	6	7
9*	**T4N1M0**	**7**	**3**
10	T4N1M0	**7**	**7**
11	T3N1M0	4	4
12	T3N1M0	7	6
13	T3N2M0	6	5
14	T4N1M0	6	5
15	T3N2M0	5	6
16	T1N1M0	2	4
17*	**T3N2M0**	**5**	**3**
18*	**T3N1M0**	**6**	**2**
19	T3N2M0	7	7
20	T3N2M0	5	7
21	T4N1M0	7	7
22	T3N1M0	6	7
23*	**T3N2M0**	**5**	**4**
24	T3N2M0	4	4
25	T3N1M0	2	0



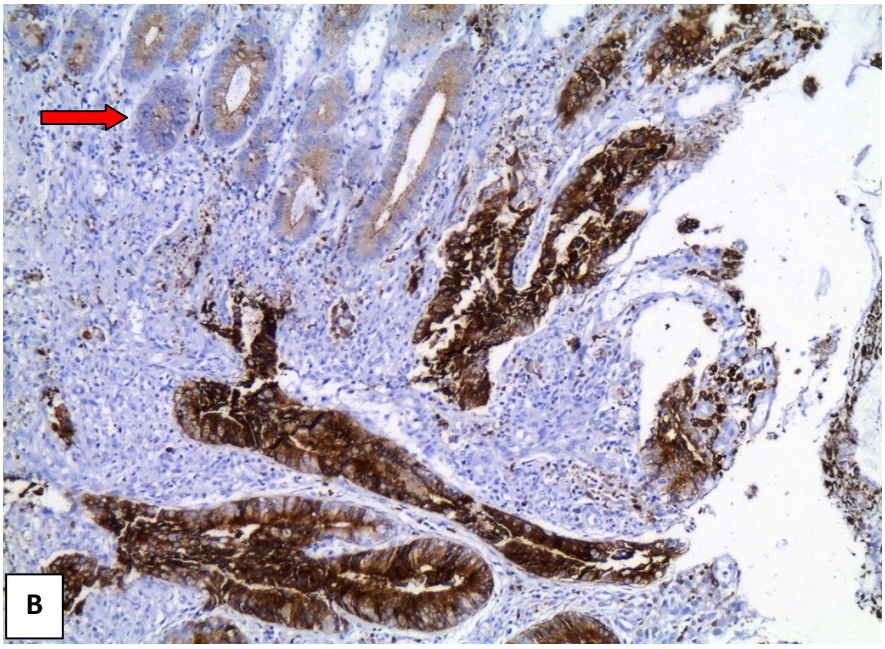


Fig 1 A : POSITIVE CONTROL: shows COX-2 expression in pulmonary adenocarcinoma, alveolar spaces are seen(red arrow)(100x). B shows strong COX-2 expression in colon carcinoma , with no expression in adjacent normal (red arrow) mucosa(100x).

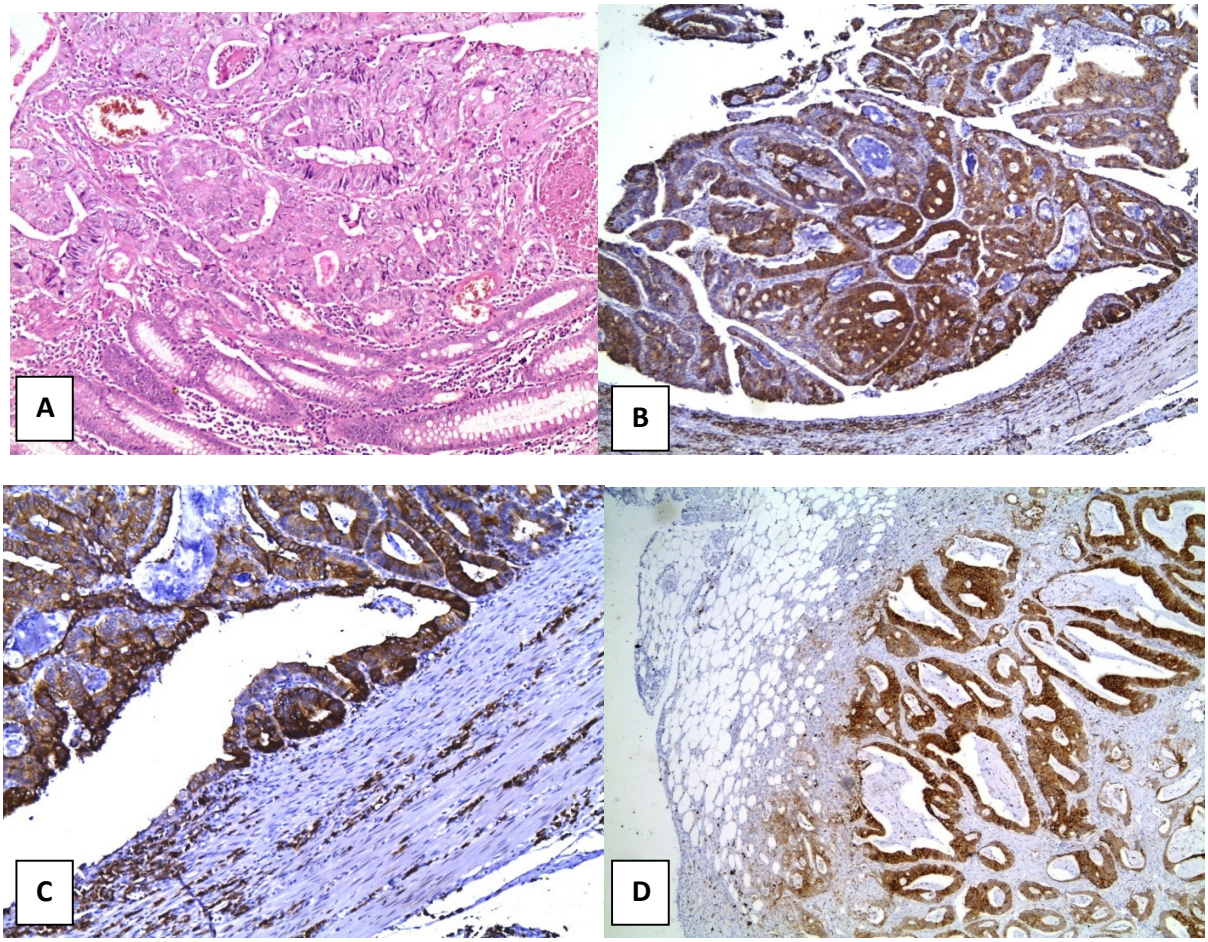


Fig 2 A : shows well differentiated adenocarcinoma (H&E, 100x). B shows strong COX-2 staining in more than 90% of tumor cells with COX2 score of (4+3)7 (40x). C shows tumor cells staining stronger than the inflammatory cells (400x). D shows strong C0X-2 expression in satellite nodule in mesocolon similar to tumor(40x).

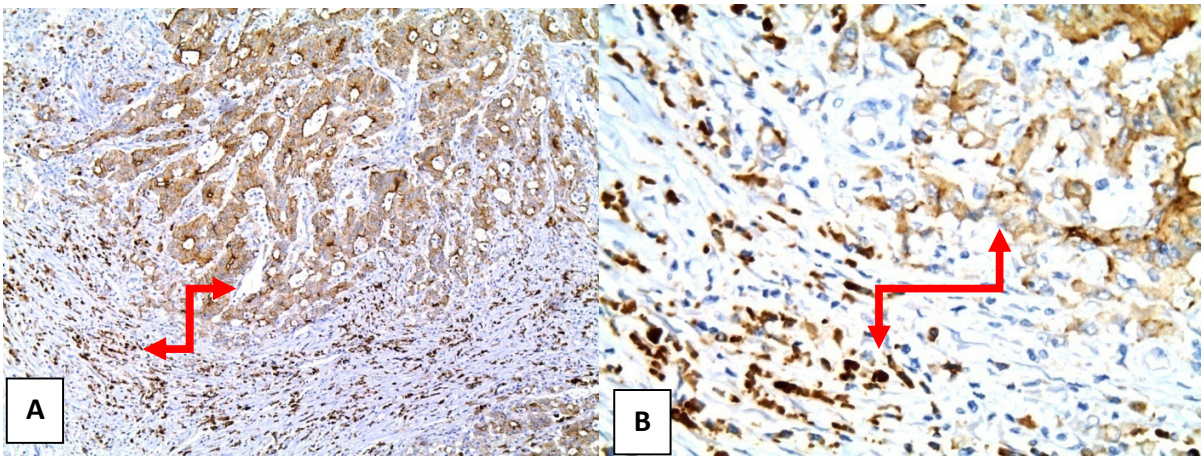


Fig 3 A shows moderate COX-2 expression of tumor cells in comparison with inflammatory cells (red arrow)(100x). B shows similar findings (400x)

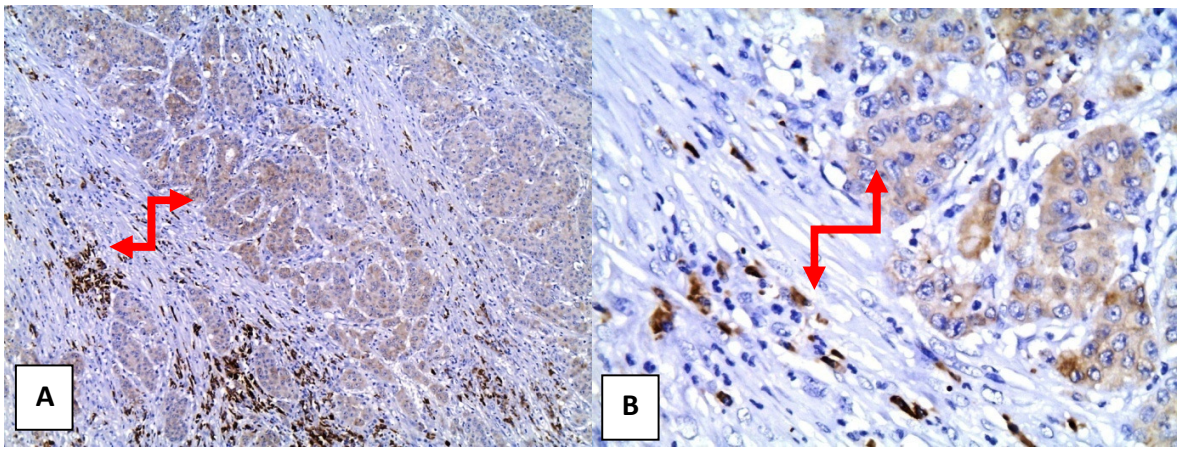


Fig 4 A shows weak COX-2 expression in tumor cells (red arrow)(100x). B shows weak COX-2 cytoplasmic staining compared to the internal positive control – inflammatory cells (400x)

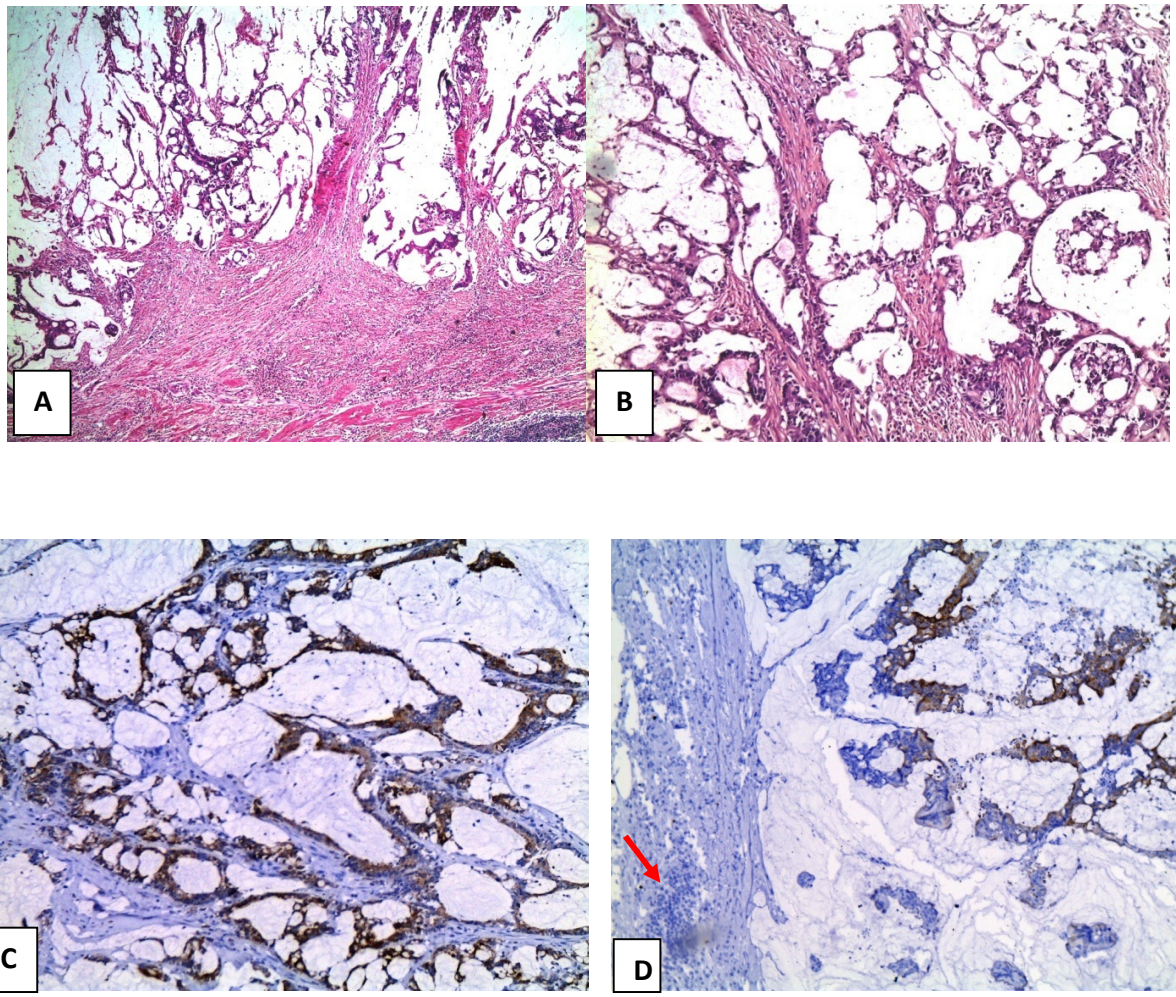
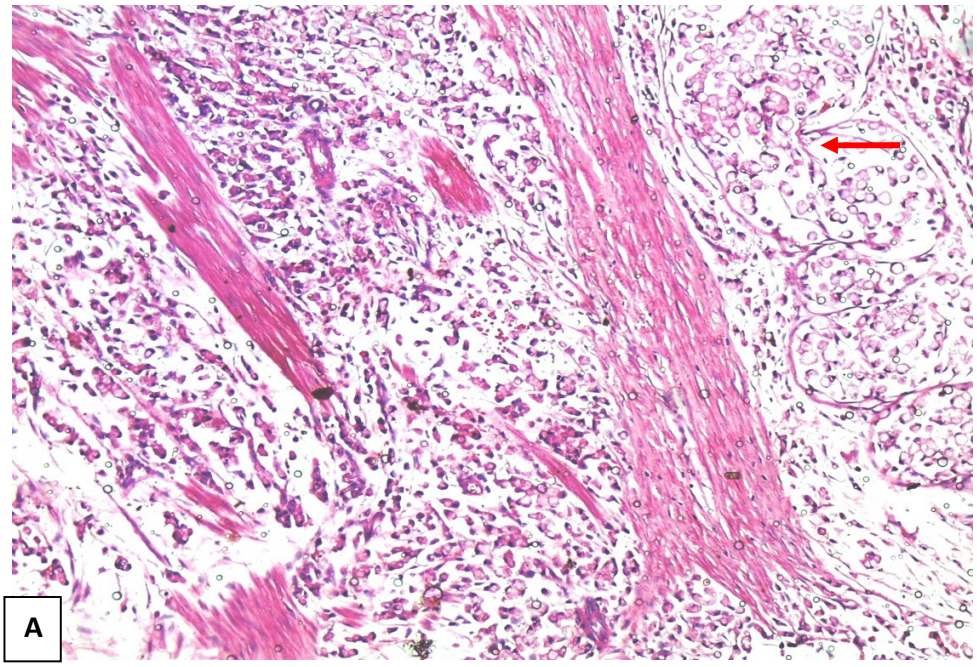


Fig 5 shows mucinous carcinoma infiltrating the submucosa with abundant extracellular mucin(H&E, 40x) .B shows tumor cells floating in pools of mucin (H&E,100x).C shows strong COX-2 expression in the same tumor(400x). D shows strong COX-2 staining in 30% of metastatic tumor cells in the lymph node amidst pools of extracellular mucin .Capsule with few lymphoid cells (red arrow)are seen(100x)



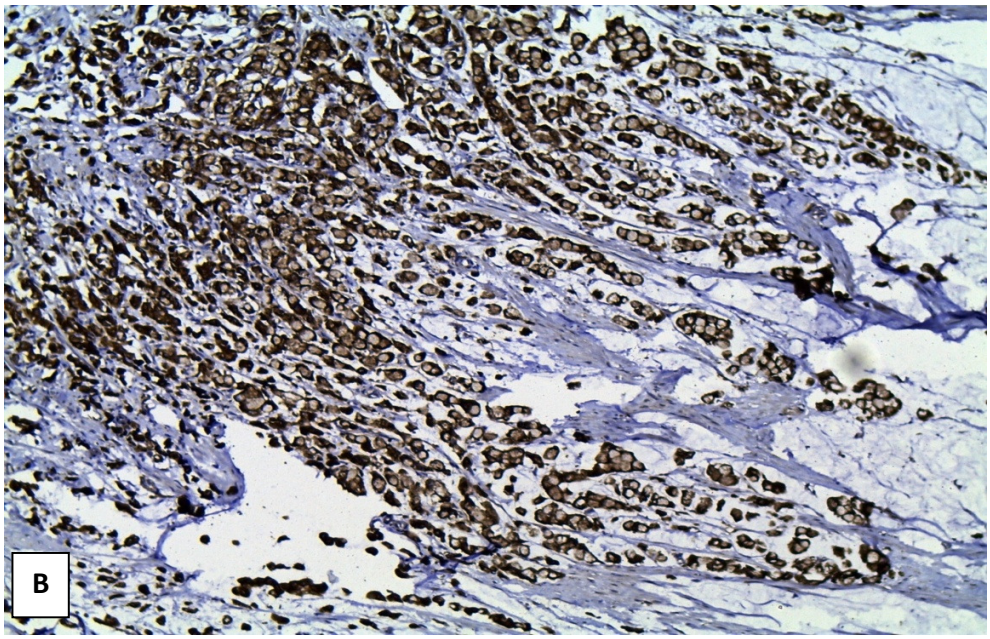
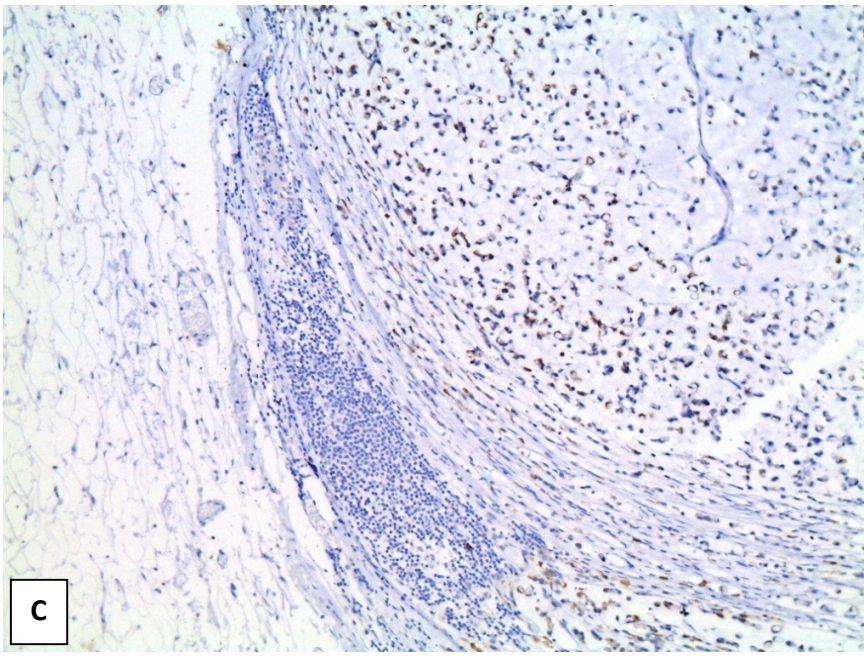


Fig 6 A shows signet ring cells (red arrow) infiltrating the muscle layer (H&E,100x).B shows strong COX-2 expression in signet ring cells(100x).



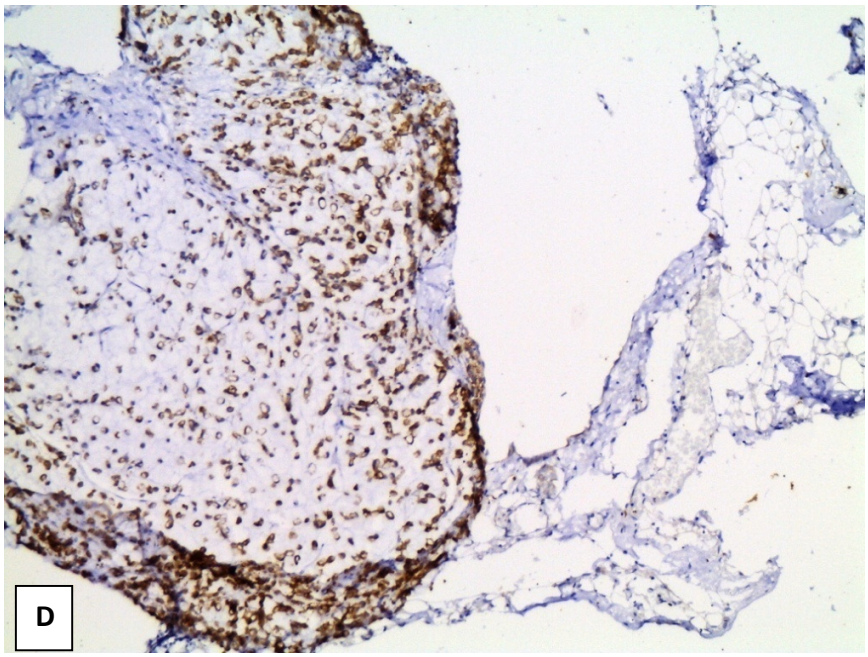


Fig 6 C shows COX-2 positive metastatic signet ring cells in lymph node (100x). D shows peritoneal nodule with tumor cells strongly expressing COX-2 (100X).

Discussion

DISCUSSION :

Colorectal cancer is the fourth most common cancer in the world and the second most common cause of cancer related death [1]. Assessment of molecular prognostic factors would be of great help for identifying patients who would benefit from adjuvant therapies, thereby increasing their survival.

Cyclooxygenase-2 (COX-2) and its product PGE_2 play an important role at multiple levels in colorectal carcinogenesis. It has been implicated from the intiation stage to tumor progression [47]. The present study was thus done to analyse the relation between COX-2 expression and the biological characteristics of colorectal carcinoma. In addition to classifying the colorectal carcinomas into a positive and a negative group, the COX-2 positive group was further divided into COX-2 low positive group which comprised of tumors with COX-2 score of 3 an 4 and COX-2 high positive group which included tumors with COX-2 score between 5 and 7 [64].

In the present study of the 65 cases, 86.2% of colorectal carcinomas expressed COX-2 while 13.8% did not express. Similar expression has been seen by Al- Maghrabi who found 85% of colorectal carcinoma expressing COX-2 [63]. However other studies have shown a relatively fewer tumors (70%) expressing COX-2 [57]. This difference could be attributed to the location of the tumor. In the former, similar to our study greater numbers of left colon

carcinoma were present, while in the latter a greater number of right colon carcinoma were included. Right colon carcinoma are more frequently associated with microsatellite instability and lower or absent COX-2 expression [67]. In agreement with these findings, 90% of left colon carcinomas were COX-2 positive while only 77.3% of right colon carcinomas expressed COX-2 in the present study.

Lymph node metastasis was present in 22.2%, 25% and 47.75% of COX-2 negative, low positive and high positive cases respectively. These findings are in corroboration with those of Sheehan KM [61] and Al-Maghrabi [63] who also found 2 to 4 times more frequent lymph node metastasis in tumors with high COX-2 expression. However the values were not statistically significant. Further as discussed above , 86.2% of primary tumors expressed COX-2 while 91.7% of the metastatic lymph nodes were COX-2 positive. A similar more frequent COX-2 expression in lymph node compared to the primary tumor has been reported by Soumaoro [57] and Al-Maghrabi [63] .

Evaluating the proportion of tumor cells expressing COX-2 , Xiong [78] and others have described strong COX-2 expression in an average of 87% of cells of primary tumor in contrast to 100% COX-2 staining in cancer cells of lymph node metastatic lesions. Such an observation was seen in the present study also. A similar COX-2 score was seen in 76% of stage III tumors in the primary tumor and lymph node tumor cells while in 4% of tumors few primary

tumor cells expressed COX-2 giving a low COX-2 score while more number of tumor cells in the lymph node expressed COX-2 and had a higher score. COX-2 expression conferring a higher metastatic potential to the tumor cells could explain the process.

With increasing stage of the tumor, there was an increase in the high COX-2 positive tumors from 33.3%, 58.8%, 80% to 100% in stage I, II, III, IV respectively. The low COX-2 expressing tumors gradually declined and there was no COX-2 negative carcinoma in stage IV. These findings are in correlation with other studies which either used the Dukes [61,62] or the AJCC staging systems [59,63], but did not reach statistically significant levels. None of the stage I tumor were COX-2 negative, but 20.6% and 8% of stage II and stage III tumors were COX-2 negative. This could be explained by the heterogenous population of right and left colon carcinomas in stage II and III as compared to the three stage I tumors which were from right, left and transverse colon.

As the tumor progressed from T1 to T4, invading the submucosa, muscularis propria, serosa and beyond, an increase in the proportion of high COX-2 positive carcinoma from 0% to 77% was observed with a decline in the low COX-2 positive tumors from T2 to T3. This association was statistically significant $p=0.021(p<0.05)$. A similar relation between depth of invasion and COX-2 overexpression has been documented by Soumaoro LT [57]and Wu AW [58] in their studies. In the present study, none of the T2 tumors were COX-2

negative, but 10.4% of T3 tumors and 23% of T4 tumors were negative as right colonic carcinomas constituted majority of the tumors of these two groups.

High COX-2 expression was seen with lesser degree of differentiation which increased from 56.2% to 66.6% and 100% in well differentiated, moderately differentiated and poorly differentiated carcinoma. In addition there was a decline in the COX-2 negative and COX-2 low-positive carcinomas with a shift towards COX-2 overexpression. Our findings are supported by Masunaga [62] and Al-Maghrabi J [63]. The values were not statistically significant.

100% of the mucinous and signet ring cell carcinomas overexpressed COX-2 (high COX-2 positive). These findings are in agreement with those by Baba Y and others [66]. A few others have described a relatively low COX-2 expression in signet ring cell carcinoma [65] compared to the mucinous category. But in the present study a high COX-2 expression was seen in the single case of signet ring cell carcinoma.

Comparing the COX-2 scores with the tumor size, in the present study no correlation was seen between the two as was seen in other studies [57,61] .Tumors with maximum diameter of 2.5cms strongly expressed COX-2 giving a total score of 7 while a few tumors as large as 7 cms in greatest diameter had a low COX-2 score of 4. In addition to the size of tumor, other morphological features such as degree of differentiation, depth of invasion and stage also determined

the COX-2 overexpression. A similar lack of association between tumor size and COX-2 overexpression has been reported by Tomozawa S [64].

Further comparing the COX-2 scores of the primary tumor and the distant metastasis in the three stage IV tumors in the study, a strong intensity of COX-2 staining was noted both in primary tumor and metastatic site in the uterus, fallopian tube, ovary, umbilical nodule giving a total COX-2 score between 5 and 7-High COX-2 expression. These features further emphasize the role of COX-2 in tumor progression.

Thus a correlation between COX-2 expression and tumor biological characters like depth of invasion (statistically significant p=0.021), stage of tumor, microscopic grade of tumor and lymph node status was seen in the present study. No correlation with tumor size was noted.

Summary and Conclusion

SUMMARY :

1 Colorectal carcinoma is the fourth most common cancer in the world, with Cyclooxygenase-2 (COX-2) and its product PGE_2 playing an important role at multiple levels in the pathway of colorectal carcinogenesis.

2 The inducible cyclooxygenase which is COX-2 is expressed constitutively only in placenta, macula densa of kidney and brain.

3 COX-2 is either absent or expressed at very low levels in the normal colonic mucosa derived from the neuroendocrine cells, macrophages and vascular endothelial cells.

4 COX-2 is induced by growth factors, cytokines, oncogenes, nuclear factor kB, IL-6 (Interleukin-6) all of which upregulate COX-2.

5 COX-2 is the enzyme that catalyzes the biosynthesis of prostaglandins from arachidonic acid.

6 COX-2 expression favours evasion of apoptosis by increasing bcl-2 expression and inhibition of Fas-mediated apoptosis. It also stimulates the APC-β catenin pathway, increases the expression of vascular endothelial growth factor (VEGF), decreases E-cadherin expression favouring epithelial-mesenchymal transition (EMT) and increases matrix metalloproteinases-2 (MMP-2) thereby enhancing the invasiveness and metastatic potential of the tumor cells.

7 The proportion of cells expressing COX-2 and the intensity of staining were both considered to calculate total COX-2 score which ranged from 0 to 7.

8 Of the 65 cases of colorectal carcinoma, 86.2% (56 cases) expressed COX-2 (COX-2 score 3 to 7) and 13.8% (9 cases) were COX-2 negative (COX-2 score < or =2).

9 12 cases (12/65=18.5%) had a COX-2 score of either 3 or 4 and were designated as COX-2 low positive. The remaining 44 cases (44/65= 67.7%) had a COX-2 score between 5 and 7 and belonged to COX-2 high positive group.

10 COX-2 expression was more frequently observed in left colon carcinoma (90%) than the right colon carcinoma (77.3%).

11 The presence of lymph node metastasis increased (22.2% to 25% to 47.7%) with the increasing levels of COX-2 expression (negative to low positive to high positive) in primary tumor.

12 A more frequent COX-2 expression (91.7%) in the metastatic lymph nodes was seen compared to the primary tumor (86.2%).

13 With increasing stage of the tumor from stage I, II, III to IV, there was an increase in the high COX-2 positive tumors from 33.3%, 58.8%, 80% to 100%.

14 As the tumor progressed from T1 to T4 invading the submucosa, muscularis propria, serosa and beyond, an increase in the proportion of tumor cells expressing COX-2 and an increase in the intensity of staining were observed thereby leading to an increase in high COX-2 positive tumors. These findings were statistically significant $p=0.021$ ($P<0.05$).

15 More frequent high COX-2 positive tumors were encountered with lesser degree of differentiation which varied from 56.2% and 66.6% to 100% in the well-differentiated, moderately differentiated and poorly differentiated carcinoma respectively.

16 Mucinous carcinoma and signet ring cell carcinomas also expressed high COX-2 score.

17 Thus in the present study COX-2 overexpression was associated with increasing lymph node metastasis, depth of invasion, stage of tumor, distant metastasis and decreasing degree of differentiation of tumor. No association was observed with tumor size.

CONCLUSION :

COX-2 the inducible isoform of cyclooxygenase expressed at sites of inflammation has recently emerged as a promising target for cancer therapy. In contrast to normal tissues in which COX-2 is either absent or expressed at very low levels, COX-2 is consitutively expressed in a variety of malignant tumors of colon, rectum, stomach, esophagus, pancreas, lung, breast and endometrium.

COX-2 is not only involved in early stages of colorectal carcinogenesis, but its rate of expression increases with the course of cancer development as seen in the present study. COX-2 expression increased with increasing stage, depth of invasion, lymph node involvement and a lesser degree of differentiation. Thus COX-2 acts at multiple levels in colorectal carcinogenesis and determines the prognosis, disease-free survival rate and the overall survival rate of the patients. These findings justify the use of a selective COX-2 inhibitor as an adjuvant to chemotherapy and radiotherapy. It gives an added advantage of increasing the response to these therapies by decreasing the bcl-2 levels, otherwise raised by COX-2.

With the advent of selective COX-2 inhibitors which do not cause gastric complications, better patient compliance can also be achieved. The newer selective COX-2 inhibitors preferred are Celecoxib, Rofecoxib, L-745,337 and SC58125 .

Bibliography

References

1.Cecilia M.Fenoglio-Preiser, Amy E.Noffsinger, Grant N. Stemmermann, Patrick E.Lantz, Peter G.Isaacson. Epithelial Neoplasms of the Colon.In: Cecilia M.Fenoglio-Preiser, Amy E.Noffsinger, Grant N. Stemmermann, Patrick E.Lantz, Peter G.Isaacson. Gastrointestinal pathology. 3rd ed. China: Raven press; 2008:593-1016.

2.Mark Redston. Epithelial neoplasms of the large intestine.In: Robert D.Odze, John R.Goldblum. Surgical pathology of the GI tract, Liver, Biliary Tract and Pancreas. 2nd ed.China:Elsevier;2009:597-637.

3.Jeremy R.Jass.Tumors of the small and large intestines (including the anal region).In: Christopher DM. Fletcher. Diagnostic Histopathology of Tumors. 3rd ed. China:Elsevier; 2007:379-412.

4.Jerrold R.Turner. The Gastrointestinal Tract. In: Vinay Kumar, Abul K.Abbas, Nelson Fausto, Jon C. Aster. Robbins and Cotran Pathologic Basis of Disease. 8th ed. India: Replika press; 2011:791-825.

5.Erhardt JG, Lim SS, Bode JC, Bode C: A diet rich in fat and poor in dietry fibre increase the in vitro formation of reactive oxygen species in human feces. J Nutr. 1997;127:706-10

6.Steinmetz KA, Potter JD: Vegetables, fruits and cancer. II.Mechanisms. Cancer causes control 1992 ; 2:427-42.

7.Newmark H, Lipkin M: Calcium, vitamin D and colon cancer. Cancer Res 1992;52(7 suppl): 2067s-70s.

8.Moskal A, Norat T, Ferri P, Riboli E: Alcohol intake and colorectal cancer risk : a dose-response meta analysis of published cohort studies. Int J Cancer 2007; 120-6.

9. Heinemann EF, Zahm SH, Mclaughlin JK, Vaught JB: Increased risk of colorectal cancer among smokers: results of a 26-year follow up US veterans and a review. Int J Cancer 1994;59:728-9

10.Potter JD. Nutrition and colorectal cancer. Cancer causes and control 1997;7:127-9

11. Yam D, Fink A, Mashiah A, Ben-Hur E: Hyperinsulinemia in colon, stomach and breast cancer patients. Cancer Lett 1996; 104: 129-31

12.Fiocchi C. Inflammatory bowel disease etiology and pathogenesis. Gastroenterology 1998;115(1): 182-205.

13.Kristina A. Matkowskyj, Zongming E. Chen, M. Sambasiva Rao, Guang-Yu Yang. Dysplastic Lesions in Inflammatory Bowel Disease, molecular pathogenesis to morphology. Arch Pathol Lab Med 2013;137:338-50.

14. Ekborn A, Helmick C, Zack M, Adami HO. Ulcerative colitis and colorectal cancer, a population based study. N Engl j med. 1990; 323(18):1228-33.

15.Mark Bettington, Neal Walker, Andrew Clouston, Ian Brown, Barbara Leggett. The serrated pathway to colorectal carcinoma: current concepts and challenges. Histopathology 2013;62:367-86.

16.Haruna Takeda, Masahiro Sonoshita, Hiroko Oshima. Cooperation of Cyclooxygenase 1 and Cyclooxygenase 2 in intestinal polyposis. Cancer Res 2003;63:4872-77.

17.Jaun Rosai. Gastrointestinal Tract- Large bowel. In: Rosai and Ackerman's Surgical pathology. 10th ed. India: Replika press;2012: 731-802

18.Hamilton SR, Vogelstein B, Kudo S, Riboli E, Nakamura S. Carcinoma of the Colon and Rectum.In: Stanley R. Hamilton, Lauri A.Aaltonen. World Health Organisation Classification of Tumors Digestive system.4th ed.Lyon: 2010;105-119.

19.Vogelstein B, Fearon ER, Hamilton SR: Genetic alteration during colorectal-tumor development. N Engl J med 1988; 319:525-32.

20.Solomen E, Voss R, Hall v: chromosome 5 allele loss in human colorectal carcinoma. Nature 1987; 328:616-18.

21.Van de Wetering M, Sanche E,Varweij C. The β–catenin/ TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell (2002);111:241-8.

22.Boland CR, Thibodeau SN, Hamilton SR. A national cancer institute workshop on microsatellite instability for cancer detection and familial predisposition development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998; 58:5248-52

23.Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007: 50;113-30.

24.Tarlakovic E, Skovlund E, Snover DC, Torlakovic G, Nesland JM. Morphologic reappraisal of serrated colorectal polyps. Am.J. Surg.pathol.2003; 27: 65-81.

25.Sullivan JN, Brunner MP, Brentall TA. Chromosomal instability in Ulcerative Colitis is related to telomere shortening. Nat Genet.2002;32(2):280-89.

26.Agoff SN, Brentnall TA, Crispin DA et al. The role of cyclooxygenase-2 in ulcerative colitis- associated neoplasia.Amj pathol.2000;157(3):737-45.

27.Graham TA, Mc Donald SA, Wright NA. Field cancerisation in GI Tract. Future oncol.2011;7(8): 981-93.

28.Makinen mj.Colorectal serrated adenocarcinoma. Histopathology 2007;50:131-50.

29.Vasse JR,Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu.Rev Pharmacol Toxicol 1998;38:97-120.

30.Bakhle YS. COX-2 and cancer: a new approach to an old problem. British Journal of pharmacology 2001;134:1137-50.

31.Williams CS, Mann M, Dubios RN. The role of cyclooxygenases in inflammation, cancer and development. Oncogene 1999;18:7908-16.

32. Cipollone F, Patrono C. Cyclooxygenase-2 polymorphism:Putting a brake on the inflammatory response to vascular injury? Arterioscler Thromb Vasc Biol 2002;22:1516-18.

33. Zidar N, Odar K, Glavac D, Jerse M, Zupanc T, Stajer D. Cyclooxygenase in normal human tissue- is COX-1 really a constitutive isoform and COX-2 an inducible isoform. J Cell Mol 2009;9:3753-63.

34. Crofford LJ. COX-1 and COX-2 tissue expression: implications and predictions. J Rheumatol suppl 1997;49:15-19.

35. Kargman S, Charleson S, Cartwright M, Frank J, Riendeau D. Characterisation of prostaglandin G/H synthase 1 and 2 in rat, dog, monkey and human gastrointestinal tracts. Gastroenterology 1996;111:445-54.

36. Gibb W Sun M. Localization of prostaglandin H synthase type 2 protein and mRNA in term human fetal membranes and deciduas. J. Endocrinol 1996;150:497-503.

37. Yamagata K, Andercasson KI, Kaufman WE, Barnes CA, Worley PF. Expression of a mitogen- inducible cyclooxygenase in brain neurons; regulation by synaptic activity and glucocorticoids. Neuron 1993;11:371-86.

38.Cao C, Matsumura K, Yamagata K, Watanabe Y. Involvement of cyclooxygenase-2 in LPS- induced fever and regulation of its mRNA by LPS in rat brain. Am J Physiol 1997;272:1712-25.

39.Hedin L, Rask K, Zhu Y, Wickman A, Wang W, Brevinge H. Role of prostaglandins in colorectal tumorigenesis: Localization and expression of COX-1, COX-2, microsomal prostaglandin E synthase-1 and the EP_2 receptor. Avicenna 2013:5 http://dx.doi.org/10.5339/avi.2013.5.

40. Yang WL, Frucht H. Cholinergic receptor up-regulates COX-2 expression and prostaglandin E2 production in colon cancer cells. Carcinogenesis 2000;21: 1789-93.

41. Brown JR, Dubois RN. COX-2: A molecular target for colorectal cancer prevention. Journal of clinical oncology 2005; 23:2840-54.

42. Knights KM, Mangoni AA, Miners JO. Defining COX inhibitor selectivity of NSAIDS: Implications for understanding toxicity. Expert Rev. Clin Pharmacol 2010;3;769-76.

43. Romero M, Artigiani R, Costa H, Oshima CTF, Miszputen S, Franco M. Evaluation of the immuno expression of COX-1, COX-2 and p53 in crohn's disease. Arq Gastro enterol. 2008;45:295-300.

44. Singer I, Kawka DW, Schlemann S, Tessner T, Riehl T, Stenson WF. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. Gastroenterology 1998;115:297-306.

45.Agoff SN, Teresa A, Brentnall, Crispin DA, Taylor SL, Raaka S et al. The role of COX-2 in ulcerative colitis- associated neoplasia. American Journal of pathology 2000;157:737-45.

46. Hendel J, Nielsen OH: Expression of COX-2 mRNA in active inflammatory bowel disease. Am J Gastroenterol 1997; 92: 1170-73.

47. Greenhough A, Smartt H JM, Moore AE, Roberts HR, Williams AC, Paraskeva C et al. The COX-2/PGE_2 pathway: Key roles in the hallmarks of cancer and adaptation to the tumor microenvironment. Carcinogenesis 2009;30:377-86.

48.Nzeako UC, Guicciardi ME, Yoon JH, Bronk SF, Gores GJ.COX-2 inhibits Fas-mediated apoptosis in cholangiocarcinoma cells.Hepatology 2002;35:552-9.

49. Hill J. Mechanisms of diet and colon carcinogenesis. Eur J cancer. Prev 1999;8:95-8.

50. Kaidi A. Direct transcriptional up-regulation of COX-2 by hypoxia-inducible factor-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. Cancer Res 2006;66:6683-91.

51.Dormond O. NSAIDS inhibit alpha V beta 3 integrin- mediated and Cdc42/Rac- dependent endothelial cell sprading, migration and angiogenesis. Nat.Med 2001;7:1041-7.

52. Wu AW, Gu J, Li ZF, Ji JF, Xu GW. COX-2 expression and tumor angiogenesis in colorectal carcinoma. World J Gastroenterol 2004;10:2323-6.

53. Metri DG, Raymond N, Bois D. Prostaglandins in cancer cell adhesion, migration and invasion. International Journal of cell biology 2012;doi:10.1155/2012/723419.

54. Tsujii M, Kawano S, Raymond N, Bois D. COX-2 expression in humen colon and cancer cells increases metastatic potential. Proc Natl Acad sci 1997;94:3336-40.

55.Ahmadi M. Prevention of both direct and cross-priming of antitumor CD8+ T cell responses following overproduction of prostaglandins E_2 by tumor cells in vivo. Cancer Res 2008;68:7520-29.

56. Basu AK, Marnett LJ: Unequivocal demonstration that malondialdehyde is a mutagen. Carcinogenesis 1983;4:331-3.

57.Soumaoro LT, Uetake H, Takagio Y, Enomoto M, Sugihara K. COX-2 expression: A significant prognostic indicator for patients with colorectal cancer. Clinical cancer research 2004;10;8465-71.

58. Wu AW, Gu J, Ji JF, Li ZF, Xu GW. Role of COX-2 in carcinogenesis of colorectal cancer and its relationship with tumor biological characteristics and patients prognosis. World J Gastroenterol 2003;9:1990-4.

59. Fujita T, Matsui M, Takaku K, Uetake H, Ichikawa W, Taketo MM et al. Size and invasion-dependent increase in COX-2 levels in human colorectal carcinomas. Cancer research 1998;58:4823-6.

60. Jun Z, Jian LJ, Zhao Z, Yao HR, Chen CY. Relationship between COX-2 expression and clinicopathological features of colorectal cancers. Chinese Medical Journal 2004;117:1151-54.

61. Sheehan KM,Sheehan K, Donoghue DP, Swuney FM, Conroy RM, Fitzgerald DJ et al. The relationship between COX-2 expression and colorectal cancer. JAMA 1999;282:1254-7.

62.Masunaga R, Kohno H, Dhar DK. COX-2 expression correlates with tumor neovascularisation and prognosis in human colorectal carcinoma patients. Clinical Cancer Research 2000;6:4064-8.

63. Al-Maghrabi J, Buhmeida A, Emam E, Syrijanen K, Sibiany A, Al-Qahtani M et al. COX-2 expression as a predictors of outcome in colorectal carcinoma. World J Gastroenterol 2012;18: 1793-9.

64. Tomozawa S, Tsuno NH, Sunami E, Hatano K, Kitayama J, Osada T. COX-2 expression correlates with tumor recurrence, especially hematogenous metastasis of colorectal cancer. British Journal of Cancer 2000;83:324-8.

65.Ogino S, Brahmandam M, Cantor M, Namgyal C, Kawasaki T, Kirkner G et al. Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component. Modern Pathology 2006;19:59-68.

66.Baba Y, Nosho K, Shima K, Goessling W, Chan AT, Kimmie NG et al. PTGER 2 overexpression in colorectal cancer is associated with microsatellite instability, independent of CpG island methylator phenotype. Cancer Epidemiol Biomarkers Prev 2010;3:822-31.

67. Nasir A, Kaiser HE, Boulware D, Hakam A, Zhao H, Yeatman T et al. COX-2 expression in right and left-sided colon cancer: a rationale for optimization of COX-2 inhibitor therapy. Chin Colorectal Cancer 2004;3:243-7.

68. Karnes WE, Shattuck- Brandt R, Burgart LJ. Reduced COX-2 protein in colorectal cancer with defective mismatch repair. Cancer Res 1998;58:5473-7.

69. Heer PD, Gosens MJEM, Bruin EC, Ensink GD, Putter H, Marijnen CAM et al. COX-2 expression in rectal cancer is of prognostic significance in patients receiving preoperative radiotherapy. Clinical Cancer Research 2007;13:2955-60.

70. Yao M, Kargman S, Lam EC, Kelly CR, Zheng Y, Luk P et al. Inhibition of cyclooxygenase-2 by Rofecoxib attenuates the growth and metastatic potential of colorectal carcinoma in mice. Cancer Research 2003;63:586-92.

71. Chan CC, Boyce S, Brideau C, Ford-Hutchinson AW, Gordon R. Pharmacology of a selective COX-2 inhibitor L-745,337: a novel NSAIDS with an ulcerogenic sparing effect in rat and non-human primate stomach. J Pharmacol Exp. Ther 1995; 274:1531-7.

72. Sandler AB, Dubinett SM. COX-2 inhibition and lung cancer. Semin Oncol 2004;31:45-52.

73.Singh B, Lucci A. Role of COX-2 in breast cancer . J Surg Res 2002;108:173-9.

74. Richardsen E, Uglehus RD, Due J, Busch C, Busund LT. COX-2 is overexpressed in primary prostate cancer with metastatic potential and may predict survival . A comparison study between COX-2, TGF- beta, IL-10 and Ki 67. Cancer Epidemiol 2010; 34:316-22.

75. Yu HP,Shi LY, Lu WH, Su YH, LiYY, Xu Sa. Expression of COX-2 in human esophageal cancer and in vitro inhibition by a specific COX-2 inhibitor NS-398. J Gastroenterol Hepatol 2004;19:638-42.

76. Ohno S, Ohno Y, suzuhi N, Soma GI, Inoue M. COX-2 expression correlates with apoptosis and angiogenesis in endometrial cancer tissue. Anticancer Research 2007;27:3765-70.

77. Jackson P, Blythe D. Immunohistochemical techniques. In: Bancroft JD, Gamble M. Theory and Practice of histological techniques.6th ed. China. Elsevier 2008:p465.

78.Xiong B, Sun IJ, Hu WD, Chang FL, Mao M, Zhou YF. Expression of cyclooxygenase-2 in colorectal cancer and its clinical significance. World J Gastroenterol 2005;11:1105-8.

Master chart

S.NO	HP NO	Age/Sex	Tumor	Max Tumor diameter	Histopathological	TNM			COX 2 SCORE					
			location	in cms	grade	STAGING	Primary tumor	Lymph node		Satellite nodule		Distant metastasis		
			R/L/T				PS +IS=TS	LP/HP	PS+IS=TS	LP/HP	PS+IS=TS	LP/HP	PS+IS=TS	LP/HP
1	332/09	70/M	L	6.5cm	Moderately differentiated	T3N0M0 IIA	4+3=7	HP						
2	400/09	42/M	L	2.5cm	Moderately differentiated	T3N2M0 IIIC	4+3=7	HP	4+3=7	HP				
3	760/09	79/M	R	6cm	Moderately differentiated	T3N0M0 IIA	3+3=6	HP						
4	1944/09	75/F	L	4cm	Moderately differentiated	T3N0M0 IIA	4+1=5	HP						
5	2386/09	69/F	R	6cm	Mucin secreting adenoca	T3N1M0 IIIB	3+3=6	HP	4+3=7	HP				
6	2432/09	72/M	L	4.5cm	Moderately differentiated	T3N0M0 IIA	2+3=5	HP						
7	2655/09	52/M	R	4.5cm	Mucin secreting adenoca	T3N0M0 IIA	4+2=6	HP						
8	2757/09	45/M	R	6cm	Poorly diff mucinous	T4N1M0 IIIB	4+3=7	HP	4+3=7	HP	4+3=7(mc)	HP		
9	2843/09	76/M	L	3.5cm	Well diff	T3N0M0 IIA	0+0=0	Neg						
10	3021/09	65/M	T	3cm	mucinous colloid type	T2N0M0 I	4+3=7	HP						
11	3196/09	43/M	L	5 cm	Well differentiated	T3N2M0 IIIC	3+3=6	HP	4+3=7	HP				
12	3209/09	47/F	L	4cm	Well differentiated	T3N0M0 IIA	1+3=4	LP						
13	3891/09	60/F	L	6cm	Well differentiated	T3N1M0 IIIB	4+2=6	HP	4+2=6	HP				
14	3912/09	48/M	L	4.5cm	Well differentiated	T3N0M0 IIA	3+3=6	HP						
15	4919/09	61/M	R	12cm	Moderately differentiated	T3N1M0 IIIB	4+3=7	HP	4=3=7	HP				
16	5011/09	56/F	R	2.2cm	Well differentiated	T2N0M0 I	3+1=4	LP						
17	5041/09	38/F	L	3.2cm	Moderately differentiated	T4N0M0 IIB	3+2=5	HP			3+3=6	HP		
18	337/10	83/M	L	4cm	Moderately differentiated	T3N0M0 IIA	4+3=7	HP						
19	883/10	55/F	L	6cm	Moderately differentiated	T4N0M0 IIB	0+0=0	Neg			1+2=3	LP		

20	979/10	74/M	T	6cm	Moderately differentiated	T3N0M0	IIA	4+2=6	HP						
21	1792/10	69/M	R	6cm	Well diff	T3N1M0	IIIB	3+1=4	LP	2+1=3	LP				
22	1938/10	63/m	L	6cm	Mucinous adenoca	T4N2M0	IIIC	3+3=6	HP	4+3=7	HP	4+3=7	HP		
23	1950/10	72/m	R	5cm	Moderately differentiated	T3N0M0	IIA	1+1=2	Neg						
24	2373/10	70/M	R	3 cm	Signet ring cell ca	T4N1M0	IIIB	4+3=7	HP	1+2=3	LP	3=6		HP	
25	2658/10	53/F	L	5cm	Moderately differentiated	T3N1M0	IIIB	1+1=2	Neg	0+0=0	Neg				
26	3817/10	76/M	R	5cm	Moderately differentiated	T4N0M0	IIB	1+1=2	Neg			1+2=3		LP	
27	3912/10	43/F	L	2cm	Moderately differentiated	T3N0M0	IIA	2+2=4	LP						
28	4340/10	63/F	L	4.5cm	Moderately differentiated	T4N1M0	IIIB	4+3=7	HP	4+3=7	HP	3+3=6		HP	
29	4542/10	71/M	R	4cm	Moderately differentiated	T4N0M0	IIB	0+0=0	Neg		1+2=3			LP	
30	4609/10	64/F	L	5cm	Moderately differentiated	T3N1M0	IIIB	1+3=4	LP	1+3=4	LP				
31	4841/10	40/F	L	4cm	Moderately differentiated	T3N0M0	IIA	4+1=5	HP						
32	45/11	78/F	R	4cm	Well differentiated	T4N0M0	IIB	4+3=7	HP			3+3=6		HP	
33	646/11	57/F	R	5cm	Moderately differentiated	T3N0M0	IIA	0+0=0	Neg						
34	2122/11	69/F	L	3cm	Well differentiated	T3N0M0	IIA	4+3=7	HP						
35	2487/11	70/M	T	6cm	Well differentiated	T3N0M0	IIA	2+3=5	HP						
36	3022/11	62/M	L	5.5cm	Moderately differentiated	T3N0M0	IIA	2+1=3	LP						
37	3233/11	58/F	L	3.5 cm	Moderately differentiated	T3N0M0	IIA	1+2=3	LP						
38	3503/11	66/F	R	2.5 cm	Well differentiated	T3N0M0	IIA	1+1=2	Neg						
39	3592/11	85/M	L	1.5 cm	Moderately differentiated	T3N1M0	IIIB	4+3=7	HP	3+3=6	HP				
40	3676/11	72/M	L	6cm	well -differentiated	T2N0M0	I	3+1=4	LP						
41	3703/11	62/F	L	5cm	Moderately differentiated	T3N2M0	IIIC	4+2=6	HP	3+2=5	HP				

42	4447/11	76/F	L	2.5 cm	Moderately differentiated	T4N0M1	IV	3+3=6	HP			4+3=7	HP	4+3=7	HP(FT)
			ovary 10.5*10*2.5 cm											4+3=7	HP(Ovary)
43	4510/11	71/M	L	4cm	Moderately differentiated	T3N0M0	IIA	1+2=3	LP						
44	4591/11	60/F	L	4cm	Moderately differentiated	T4N1M0	IIIB	3+3=6	HP	4+1=5	HP	3+3=6	HP		
45	5051/11	77/M	L	5cm	Moderately differentiated	T3N0M0	IIA	1+2=3	LP						
46	5091/11	60/M	L	6cm	Moderately differentiated	T3N2M0	IIIC	3+2=5	HP	4+2=6	HP				
47	626/12	61/F	L	5cm	Well differentiated	T1N1M0	IIIA	1+1=2	Neg	3+1=4	LP				
48	685/12	85/F	L	5cm	Moderately differentiated	T3N0M0	IIA	3+1=4	LP						
49	1289/12	69/F	L	5cm	Moderately differentiated	T4N0M1	IV	4+2=6	HP			3+3=6	HP	4+1=5	HP-Uterus
			uterus 1.6*1.5*1.2cm											4+2=6	HP-FT
														3+2=5	HP-Ovary
50	1675/12	93/M	R	8cm	Moderately differentiated	T3N0M0	IIA	4+2=6	HP						
51	2134/12	59/M	R	8cm	Moderately differentiated	T3N0M0	IIA	4+2=6	HP						
52	2513/12	46/M	L	4cm	Moderately differentiated	T3N2M0	IIIC	3+2=5	HP	2+1=3	LP				
53	2548/12	79/F	R	9cm	Moderately differentiated	T4N2M1	IV	4+3=7	HP	4+3=7	HP	4+3=7	HP	4+3=7	HP
54	3160/12	61/F	L	6cm	Moderately differentiated	T3N1M0	IIIB	3+3=6	HP	1+1=2	Neg				
55	3176/12	77/M	L	4.5cm	Moderately differentiated	T3N0M0	IIA	3+2=5	HP						
56	3437/12	70/M	R	7cm	Well differentiated	T3N0M0	IIA	2+3=5	HP						
57	4532/12	67/F	L	6cm	Moderately differentiated	T3N2M0	IIIC	4+3=7	HP	4+3=7	HP				
58	4627/12	55/M	L	5.5cm	Well differentiated	T3N2M0	IIIC	3+2=5	HP	4+3=7	HP				
59	4713/12	41/M	R	9.5cm	well differentiated mucin	T4N1M0	IIIB	4+3=7	HP	4+3=7	HP	4+3=7	HP		
60	4959/12	85/F	L	5cm	Well differentiated	T3N1M0	IIIB	3+3=6	HP	4+3=7	HP				

61	184/13	56/M	L	4.5cm	Moderately differentiated	T3N0M0	IIA	4+3=7	HP						
62	256/13	45/F	L	5cm	Moderately differentiated	T3N2M0	IIIC	2+3=5	HP	3+1=4	LP				
63	698/13	56/F	R	7cm	Moderately differentiated	T3N2M0	IIIC	3+1=4	LP	4+1=5	HP				
64	1018/13	61/M	R	6cm	Moderately differentiated	T3N0M0	IIA	3+3=6	HP						
65	1885/13	79/M	R	8cm	Moderately differentiated	T3N0M0	IIA	3+3=6	HP						